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PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS,

IN SESSION IN

Boston, December 28–30, 1909.

SCIENTIFIC PROGRAM

EDITED BY THE SECRETARY.

Summary of Meetings.

PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

First Meeting

Tuesday morning, December 28. Harvard Medical School. Presiding Officer: the President, Otto Folin.

SOME POINTS IN THE ANALYSIS OF PROTEINS.

By THOMAS B. OSBORNE AND D. BREESE JONES.

(From the Connecticut Agricultural Experiment Station, New Haven.)

Loss may result as follows:

(I) Through incomplete hydrolysis, for resistant peptid combinations of amino-acids may occur which require more energetic treatment to decompose them than has heretofore been employed.

(II) Through formation of humin, a secondary decomposition product, which represents a loss greater than its own quantity. Zein yields only traces of humin hence this does not originate from any of the amino-acids yielded by this protein.

(III) Through incomplete separation of glutaminic acid which cannot be as perfectly isolated as is commonly supposed. Leucin hydrochlorid may separate with glutaminic acid hydrochlorid and interfere with the separation in a state of purity.

(IV) Through incomplete esterification. The method of Phelps and Tillotson has yielded results much higher than those by Fischer's method and thereby a large part of the previously observed deficiency has been accounted for.

Analysis of a mixture of pure amino-acids in the same proportion as they were obtained from zein showed that the proportion of the several amino-acids recovered ranged between 41 and 82 per cent of the amount originally present in the mixture. Assuming like losses to occur in analyzing zein the estimated total accounted for would equal 80 per cent of the zein, assuming the

amino-acids to be in polypeptid union and the ammonia as united with one carboxyl of the dibasic acids. The 14 per cent deficit may be due to incomplete hydrolysis and to secondary decomposition, but as yet no definite data have been obtained on this point.

ON THE PENTOSE IN THE PANCREATIC GLAND.

By W. A. JACOBS and P. A. LEVENE.

(From the Laboratories of the Rockefeller Institute for Medical Research.)

In the work already published by us on inosinic, guanylic and the yeast nucleic acids we have definitely proven that the pentose obtained from these acids in pure crystalline form is *d*-ribose. We incidentally showed that the conclusion arrived at by Neuberg that this sugar is *l*-xylose is untenable. It has been generally accepted that guanylic acid is the sole pentose-containing constituent of the pancreas nucleoprotein. We therefore considered that *d*-ribose was the only pentose contained in the nucleoprotein. But the possibility that *l*-xylose might be present in some other combination in the gland was not excluded, which, if so, would explain the results of Neuberg. A priori, however, it seemed unlikely that *l*-xylosazon could be obtained from this source without being contaminated by the *d*-ribosazon which has similar solubilities. To definitely decide this point we extended our investigations to the gland itself. The procedure was much the same as that employed by Salkowski, and later in his own work by Neuberg. The pentosazon obtained by us possessed all the properties of the *d*-ribose derivative. From the guanylic acid obtained from the liver nucleoprotein we also obtained the osazon of the same sugar. On the basis of these results we consider ourselves justified in concluding that *d*-ribose is the only sugar of the pentose group occurring in the nucleoproteins of the pancreas and the liver.

THE METABOLISM OF PYRIMIDIN DERIVATIVES.

By LAFAYETTE B. MENDEL and VICTOR C. MYERS.

(From the Sheffield Laboratory of Physiological Chemistry in Yale University.)

The pyrimidin derivatives, uracil, cytosin, and thymin, were introduced both parenterally and orally into a variety of animals

and man in order to study the behavior of these compounds in metabolism. Although pyrimidins could not be isolated in detectable amounts from the urine after feeding typical nucleoproteins containing them, the introduction of their characteristic pyrimidin derivatives was regularly followed by the elimination of them in larger or smaller quantity through the kidneys. They are not completely transformed, as are the purins under similar conditions, but may be recovered in noteworthy amounts. Administration of uracil, thymin, and cytosin was not followed by any increased output of purin derivatives (with a possible exception in the case of thymin administration in rabbits)—thus giving no evidence of a synthesis of purins directly from pyrimidins in the animal body. They have no influence on the output of creatinin (or creatin). Contrary to the deductions drawn by others, thymin and some other methylated pyrimidins were not found to exhibit diuretic properties; nor did any of the pyrimidins studied exert any noteworthy effects on metabolism. Experiments with liver extracts failed to reveal the existence of enzymes capable of transforming the preformed pyrimidins in ways comparable with those ascertained for the purin compounds.

FURTHER OBSERVATIONS ON THE NITROGEN BALANCE IN PREGNANT DOGS.

By J. R. MURLIN.

(From the Physiological Laboratory of the Cornell University Medical College, New York City.)

Two new experiments made on the same dog beginning in each case several weeks previous to the menstrual period and continuing for three weeks into the gestation period show that the amount of nitrogen loss is not greater in the early weeks of gestation than it is in complete sexual rest. The metabolism during the menstrual period, however, shows a marked retention of nitrogen, as already reported by Schöndorff for dogs and by Schrader for women.

THE PURIN ENZYMES OF THE GUINEA-PIG AND RABBIT.

By PHILIP H. MITCHELL.

(From the Biological Laboratory of Brown University, Providence, R. I.)

Since it has been shown that the purin enzymes, nuclease, adenase, guanase, oxidase and uricase, occur with different distribution in the different organs of the same animal and in the same organs of different animals, the question arose as to whether guinea-pigs and rabbits, slightly but not closely related species possess the same distribution of purin enzymes.

Some data concerning tissues of rabbits already exist and uricase has been identified in the liver of the guinea pig. In this research further experiments have been performed with organs of the rabbit; and liver muscles, kidneys and stomach walls of the guinea pig have also been compared for their content of adenase and guanase while the liver and muscles were tested for oxidase and uricase. In every case the comparison has shown that the tissues examined in the guinea pig and the rabbit exhibit the same enzymotic power toward purin compounds.

These results suggest the possibility that the relationships of species may be indicated by the distribution of purin enzymes. Further experiments are in progress.

SOME ANCESTRAL FEATURES IN THE BLOOD PLASMA OF VERTEBRATES.

By A. B. MACALLUM.

(From the Biochemical Laboratory of the University of Toronto.)

Analyses of the inorganic constituents of the blood plasma in Teleosts (cod and pollock) and Elasmobranchs (dog fish and sand shark) show a composition almost parallel to that of the blood plasma of mammals. The *Gadidæ* have been marine forms since the beginning of the Tertiary, while the Elasmobranchs have been oceanic since their origin in the early Paleozoic, and though there is in their blood plasma an increase in the total quantities of the sodium, potassium, magnesium and calcium over those in mammalian blood plasma, the relative proportions between these

are not strikingly different from those found in mammals. It would appear from this that these proportions are of a common origin, probably derived from those of the blood of Protovertebrate stock, and these in their turn from a more ancient source, i.e., Pre-Cambrian sea-water.

NOTE ON THE USE OF CHITIN IN DIALYSIS.

By CARL L. ALSBERG.

(From the Laboratory of the U. S. Bureau of Fisheries at Woods Hole, Mass.)

The behavior of chitin as a dialyzing membrane is of some interest because as the covering of the gills of many invertebrates it may perhaps play a part in the gas and salt metabolism of these animals. It may be used instead of collodion or parchment paper for many purposes, as it lets some substances diffuse through while holding back many others with a larger molecule. It offers the advantage of being unaffected by the strongest alkalies and by all but very concentrated acids, as well as that of having a relatively great tensile strength. To prepare it for use it is freed from protein by caustic alkali, decalcified, washed free from acid and boiled out with alcohol. If the end of a lobster's claw be prepared in this way a very strong seamless sack is obtained very convenient for dialyzing small quantities. From the carapace of the horseshoe crab quite large sheets may be obtained. There is some evidence that chitin exerts some selective action, a phenomenon that is still under investigation.

THE ESTIMATION OF UREA.

By STANLEY R. BENEDICT.

(From the Laboratory of Physiological Chemistry, University of Syracuse.)

Urea has the specific property of being hydrolyzed through the action of salts in the presence of minimal amounts of water. Where water is present in excess, uric acid and creatinin are hydrolyzed in considerable amount. The Folin method owes its accuracy to combining two conditions found in no other method so far proposed, viz: (1) the hydrolyzing agent is a salt, and (2) the hydrolysis takes place in the practical absence of water. The

Benedict-Gephart method does not combine these conditions, and the autoclave procedure is therefore, less accurate, in any possible modification, than is the Folin method.

The Folin method is not, however, absolutely accurate. It yields about 0.3 per cent nitrogen from creatinin, 2 per cent of nitrogen from uric acid, and 100 per cent of nitrogen from allantoin. The percentage decomposition of creatinin and uric acid by the Folin process is, however, so small as to be of no significance in urine work. The details of this work, including a new method for urea estimation based upon heating the urinary residue in an open tube with certain salts, will shortly be published.

THE USE OF INVERTASE IN THE DETERMINATION OF THE ALKALINITY OR ACIDITY OF BIOLOGICAL FLUIDS.

By C. S. HUDSON AND WILLIAM SALANT.

(From the Bureau of Chemistry, United States Department of Agriculture.)

Invertase prepared from yeast loses most of its activity when it is dialyzed; this is caused by the loss of acids which pass through the membrane, since the original activity reappears when new acid is added. When invertase is in very weakly acid solution the addition of alkali lowers its activity but added acid increases it. It is thus a very sensitive indicator. Its activity in pure water being 300 (arbitrary units), it was found that in bloods its activity was as follows: dog's, 266; cat's, 198; cow's, 107; frog's, 88; sheep's, 82; pig's, 69, indicating that the sensitiveness of invertase to these bloods is very great. If the blood is dialyzed it loses its power of lowering the activity of invertase, showing that the lowering is due to the salts of the blood.

NOTE ON THE DETERMINATION OF KREATININ.

By P. A. SHAFFER AND E. A. REINOSO.

(From the Laboratory of Chemical Pathology, Cornell University Medical School, New York.)

With urine or other solutions which contain less than 20 mg. of kreatinin in 100 cc., the Folin method does not yield correct results.

The following modifications of Folin's directions yield the correct results in solutions of any concentration exceeding about 3 mg. in 100 cc.

Use for the determination such a volume of the urine or other solution as will contain between 8 and 12 mg. of kreatinin. If the volume used be less than 225 cc. add an equal volume of saturated picric acid and one-tenth of this volume of 10 per cent NaOH. Otherwise follow the original directions. As examples see the following:

Constant amount of kreatinin.

VOL. K. SOLN.	PICRIC ACID	10 PER CENT NaOH	Mg K FOUND
10	15	5	8.18
100	15	5	5.02
100	100	10	8.10
200	200	20	8.10

With still more dilute solutions one may add a known amount of kreatinin and determine the unknown by difference.

One must however, remember that if the kreatinin solution is colored, this color will probably interfere, and if possible should be removed when a large volume of the solution is taken for the determination.

IS THE BENCE JONES PROTEIN PRODUCED FROM OSSEOALBUMOID?

By JACOB ROSENBLOOM.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

In continuation of the work inaugurated in this connection by Ottenberg and Gies[1] the author has conducted an investigation with results that warrant the following conclusions:

(1) The peptic digestion of osseoalbumoid yielded a water soluble substance precipitable by saturation with ammonia sulfate.

[1] Ottenberg and Gies: *Proc. Soc. Exp. Biol. and Med.*, iv, p. 161, 1907.

In one instance this material gave a heat precipitation test, but the reaction was not as marked in its sharpness as the same test when applied to elastose. Negative results were obtained when the test was applied to two other similar products produced in the peptic digestion of osseoalbumoid.

(2) After the subcutaneous injection of the material produced by the peptic digestion of osseoalbumoid, the urine did not yield a heat-precipitation test, but gave a positive reaction with picric and trichloracetic acids. The $(NH_4)_2SO_4$ precipitate from the urine in this experiment gave positive heat as well as picric acid and trichloracetic acid tests.

(3) The heat precipitation test in the urine is determined by the amount of substance excreted as well as by the reaction of the urine and the quantity of salts present.

(4) After the subcutaneous injection of the material produced by the peptic digestion of osseoalbumoid, an alcoholic precipitate of the substance excreted in the urine is rendered insoluble on standing under alcohol, thereby showing one of the characteristics of the Bence Jones protein, and departing from the usual properties of proteoses.

(5) After subcutaneous injection of the material obtained by the tryptic digestion of osseoalbumoid, it was excreted in the urine but it did not give a heat-precipitation test.

(6) Osseoalbumoid undergoes proteolysis when subjected to the action of the enzyme of leucocytes that acts in an alkaline medium. A solution of the $(NH_4)_2SO_4$ precipitate of this digestive mixture gave a heat precipitation test as well as reactions with picric acid and trichloracetic acid.

(7) The material produced by leucocytic proteolysis of osseoalbumoid, after its subcutaneous injection, was found to have been modified in the body and was excreted, in whole or in part, as a nucleoalbumin-like substance in the urine.

(8) Intraperitoneal injection could not be used as a satisfactory method of favoring autolysis of osseoalbumoid on account of the peritonitis produced.

(9) Osseoalbumoid was absorbed, after its intrapleural injection, and a digestive product of it was excreted in the urine. This product in the urine did not give a heat-precipitation test, but a solution of the $(NH_4)_2SO_4$ precipitate from the urine

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gave this reaction as well as one with picric acid and trichloracetic acid.

(10) Some of the results indicate that the Bence Jones protein *may be* formed from osseoalbumoid by the action of enzymes present in the bone marrow.

The work of the kidney as a regulator of the reaction of the blood and protoplasm. Lawrence J. Henderson.

The isolation of pepsin-proteoses with high proportions of basic nitrogen. Andrew Hunter.

Second Meeting.

Wednesday morning, December 29. Harvard Medical School. Joint session with the American Physiological Society. Presiding officer: The president of the American Physiological Society, William H. Howell.

THE ACTION OF CERTAIN SUBSTANCES UPON THE RESPIRATORY CENTER.

By A. S. LOEVENHART and W. E. GROVE.

(From the Pharmacological Laboratory of the University of Wisconsin.)

For some time we have been engaged in the study of

Sodium o. iodobenzoate, $C_6H_4\left\langle\begin{matrix} I \\ COONa\end{matrix}\right.$

Sodium o. iodosobenzoate, $C_6H_4\left\langle\begin{matrix} I=O \\ COONa\end{matrix}\right.$

Sodium o. iodoxybenzoate, $C_6H_4\left\langle\begin{matrix} I\left\langle\begin{matrix} O \\ O\end{matrix}\right. \\ COONa\end{matrix}\right.$

The salts were injected intravenously into dogs, cats, and rabbits.

We have found that sodium iodosobenzoate cannot oxidize phenolphthalin to phenolphthalein unassisted, but that it does so

in the presence of blood serum. In other words, sodium iodosobenzoate can furnish oxygen for the peroxidase reaction, behaving in this respect like hydrogen peroxide. We have taken this fact as evidence that the oxygen bound to the iodine in iodosobenzoic acid is physiologically active and capable of being utilized by the body for purposes of physiological oxidations.

On injecting minimal doses (1 to 2 cc.) of $\frac{N}{100}$ solution of sodium iodosobenzoate or oxyiodosobenzoate we noted an immediate and marked depression of the respiratory center, a center which is notably very sensitive to changes in the oxidative processes. The depression seems to be identical with ordinary apnea produced by ventilation, and recovery from it is spontaneous. The duration of the complete apnea depends on the dose and may last from two to three or, in rare cases, four minutes. Iodoxybenzoic acid seems more powerful than iodosobenzoate in producing the apnea and also contains a higher percentage of active oxygen. Iodobenzoic acid was found to be entirely inactive in the doses employed by us. It is of great interest to note that the taste of sodium iodosobenzoate is indistinguishable from that of hydrogen peroxide, and we believe that the very characteristic taste of both substances is due to the action of the active oxygen on the end organs of taste.

LIPASE.

By HAROLD C. BRADLEY.

(From the Laboratory of Physiology of the University of Wisconsin.)

Human pancreatic lipase in its action on triolein demonstrates some of the points of departure of enzymes from true catalytic agents. The amount of lipase present in a digestive mixture determines the extent of digestion, i.e., the point of final equilibrium between the triolein and the products of its hydrolysis. When lipase is relatively abundant digestion is practically complete. When the amount of triolein is great in proportion to the lipase the percentage hydrolysis is small, though the actual amount of acid liberated may be considerable. The points of pseudo-equilibria may thus be varied from 0 per cent to 100 per cent digestion by suitably relating enzyme to zymolite. The pseudo-equilibria are usually assumed to be due to the destruc-

tive action of the fatty acid upon the lipase. If this be true it is evident that the reaction cannot be reversible under these conditions, for the free acid would at once destroy the lipase; if the enzyme were not destroyed the digestion would remain at 100 per cent hydrolysis, as it does when a sufficient amount of enzyme is added to neutral oil. Fat synthesis in the body must therefore depend upon other factors than reversibility of lipase reactions.

THE ACTION OF THE BLOOD PROTEINS ON THE ISOLATED MAMMALIAN HEART.

By L. W. GORHAM and A. W. MORRISON.

PRESENTED BY W. H. HOWELL.

(*From the Physiological Laboratory, Johns Hopkins University.*)

This paper gave an account of experiments made upon the isolated heart of the cat. The heart was thoroughly irrigated for an hour and kept in living condition by a Locke's solution fed to the coronary arteries through the aorta (Martin's method). After all traces of blood had been removed from the heart it was fed in the same way with a small amount of Locke's solution containing a known concentration of fibrinogen, serum globulin or serum albumin. This small amount was circulated through the heart repeatedly for several hours, and at the end a quantitative estimate was made of the concentration of the protein to ascertain whether or not there had been a change. The general result of the experiments indicated that circulation through the living heart is followed by a disappearance of fibrinogen and of serum globulin, while the serum albumin is not affected. The experiments were interpreted as showing in the first place a marked difference in reaction between the globulins and the albumins of the blood, and secondly as indicating a possible direct nutritive function of the globulins. The paper with all details of methods and results has been published in the *American Journal of Physiology* (xxv, p. 419, 1910).

THE ABSORPTION OF FLUID FROM THE PERITONEAL CAVITY.

By MOYER S. FLEISHER and LEO LOEB.

(From the Laboratory of Experimental Pathology of the University of Pennsylvania.)

The mechanism through which substances are absorbed from the peritoneal cavity under various conditions is not yet well understood.

In continuation of former experiments in which we studied the elimination of fluid into the peritoneal cavity under various conditions, we have carried out an extensive series of experiments on approximately 420 rabbits, studying the conditions that influence the rate of absorption from the peritoneal cavity. We investigated the influence of the osmotic pressure, of salt solution, the influence of adrenalin, furthermore the influence of uranium nitrate and of ligature of the renal vessels and certain other operative interferences in other parts of the body. We also included a study of the effect of caffein and of various combinations of these factors upon absorption. If we exclude the action of uranium nitrate and of peritonitis (by which complicating factors certain changes in the membranes separating the peritoneal fluid from the surrounding tissues and blood, probably are produced), in all the other conditions in which we made the appropriate tests we have been able to foresee their influence upon the rate of absorption by determining their effect on the osmotic pressure of the blood. *Vice versa* it was possible to foretell the effect of those conditions upon the osmotic pressure of the blood by studying their influence upon absorption. A few examples may be given.

Nephrectomy or ligature of the renal vessels causes increased osmotic pressure of the blood and an increased rate of absorption from the peritoneal cavity.

But other operations as for instance an incision into the skin and muscle influence the osmotic pressure of the blood as well as absorption approximately in the same way as nephrectomy although some insignificant quantitive differences may exist. This cannot, therefore, be a specific effect upon the kidney.

Narcosis by means of ether alone, however, affects neither the osmotic pressure of the blood nor the rate of absorption.

Adrenalin increases the rate of absorption of 0.85 per cent NaCl solution as well as that of a hypertonic, for instance, 1.5 per cent NaCl solution and also the rate of absorption of distilled water. Correspondingly, adrenalin increases the osmotic pressure of the blood. In these latter experiments adrenalin was injected intraperitoneally.

Under the influence of caffein the osmotic pressure of the normal rabbits is not raised but rather somewhat lowered. Correspondingly we find that absorption of fluid from the peritoneal cavity is not increased under the influence of caffein.

In nephrectomized animals on the other hand a noticeable rise in the osmotic pressure of the blood is produced under the influence of caffein and correspondingly we find that caffein increases markedly the absorption of fluid from the peritoneal cavity.

We notice thus an inverse action of caffein upon absorption of fluid and upon diuresis.

These experimental conditions not only influence the distribution of fluid in the body but also the distribution of the chlorides and of other osmotically active substances and these changes do not usually take place in a parallel direction.

THE PRODUCTION OF SUGAR FROM AMINO-ACIDS.

By A. I. RINGER and GRAHAM LUSK.

(*From the Laboratory of Physiology, Cornell Medical College, New York City.*)

Various amino-acids were given to dogs with total phlorhizin glycosuria. The results show that both glycocoll and alanin may be completely converted into sugar, and that three carbon atoms of the four which are contained in aspartic acid and also three of the five contained in glutamic acid are convertible into dextrose. The results are given in the following table:

Results in grams after ingestion of 20 grams of substance.

		Glycocoll	Alanin	Aspartic acid	Glutamic acid
N ingested	a	3.77	3.12	2.14	1.95
	b	3.77	3.12	2.14	1.95
Extra dextrose eliminated	a	13.97	18.76	11.32	13.09
	b	15.71	18.78	12.26	13.46

Theoretical amount in grams of dextrose that might be made from:

2 C atoms....................	16		9.02	8.16
3 C atoms....................		20. 2	13.56	12.24
4 C atoms....................			18.04	16.32
5 C atoms....................				20.40

FURTHER STUDIES ON THE INTERNAL SECRETION OF THE THYROIDS AND PARATHYROIDS.

By A. J. CARLSON and A. WOELFEL.

(From the Hull Physiological Laboratory of the University of Chicago.)

The work so far has been directed mainly towards discovering the presence of the secretion in the thyroid lymph of dogs with goitres.

(1) Lymph production in normal thyroids is very small. It increases greatly in all forms of thyroid growth, and proportionally to the growth or increased size. This applies also to tumors of the thyroid. In our experience the most copious lymph flow was secured from two cases of carcinoma of the gland.

There appears to be no relation between the percentage of iodine in the thyroid and the rate of lymph production. Goitres in which no iodine could be detected exhibit just as copious lymph production as the goitres rich in iodine. The rate of lymph production appears to be correlated with the growth processes and the actual size of the gland.

(2) Chemical test for the internal secretion. All our tests for iodine in goitre lymph (quantities from 20 to 200 cc.) have been negative.

(3) Physiological tests for the internal secretion. Intravenous injections of goitre lymph in normal dogs usually produce a rise of temperature, irregularity of the heart, and occasionally tremors. These symptoms disappear in 8 to 20 hours. The same symptoms are, however, produced by intravenous injections of equal quantities of blood from the same animal. These phenomena are therefore not specific, and do not show thyroid secretion in the thyroid lymph.

(*a*) Intravenous injections of goitre lymph in dogs under anesthesia have given in 40 per cent of our experiments a gradual

depression of the blood pressure accompanied by cardiac irregularity, partly due to action on the vagi centers in the medulla. The heart actions suggest hyperthyroidism, but the fact that the results are not always obtained renders such a conclusion extremly insecure.

(*b*) The Hunt aceto-nitrile test has been made on goitre lymph from six dogs. The mice were fed 1 cc. of the lymph daily for 6 to 10 days. In no case was any increased resistance noted. The quantities fed may not have been great enough; the lymph may not have contained any of the secretion; or the Hunt test is a test for iodine rather than for thyroid secretion.

(*c*) Complete elimination of the neck and thyroid lymph for 48 hours in six normal foxes. The results were negative, the animals showing no symptoms whatever. Thyroid insufficiency symptoms would probably not have appeared within that period even if all the internal secretion had been eliminated, but symptoms of parathyroid insufficiency ought to have been in evidence, if the secretion of that gland reached the blood by way of the lymphatic lymph.

(4) The tests for the thyroid-parathyroid secretions in the lymph are therefore all negative so far, and will scarcely permit any positive conclusion. But in the light of other work on lymph physiology recently completed, it seems probable that when adequate tests for the thyroid-parathyroid secretions are developed these secretions will be found in greater concentration in the blood than in any of the lymphs, and that they enter the blood directly rather than indirectly by way of the lymphatic lymph.

THE RELATION OF PTYALIN CONCENTRATION TO THE DIET AND TO THE RATE OF SALIVARY SECRETION.

By A. J. CARLSON and A. L. CRITTENDEN.

(*From the Hull Physiological Laboratory of the University of Chicago.*)

1. *The Relation of Ptyalin Concentration to the Diet.*

(*a*) In Man. In the fall of 1908 the diastatic power of the parotid saliva of three individuals (A. J. C., A. L. C., C. C.)

designated for convenience as A, B and C, was compared daily for a period of ten days. The saliva of A was uniformly slightly stronger than that of B, and considerably stronger than that of C. At the end of the ten day period B and C were put on an exclusive vegetarian diet, that is, meat was excluded and the carbohydrates greatly increased, for ten days, while A continued on the ordinary mixed diet. The diastatic power of the parotid salivas was tested daily and *there was no increase in B and C as checked against A.*

(2) The diastatic power of the parotid saliva of a man who for four years has been a consistent vegetarian was checked (for seven days) against that of A and B. *It was uniformly less than A and practically the same as B.*

(3) The parotid and mixed saliva of a boy of 14 years, a "congenital" vegetarian, never eating meat, was checked against the corresponding salivas of A and B. It showed *uniformly less diastatic power than A, and about the same as B.*

Thus, contrary to Neilson's results, there is no evidence that in man *even years of exclusion of meats and greatly increased carbohydrates in the food will appreciably increase the ptyalin concentration in the saliva.*

(*b*) In Other Mammals. It is conceivable that while shorter periods of meat exclusion and carbohydrate increase in the diet of man may not effect an increase in the ptyalin, generations of vegetarianism might be effective. This could be tested on the saliva of orthodox Hindoos, but we were not able to secure this material. The experiment has, however, been carried out in nature on a large scale in the case of the herbivora.

(1) Carnivora: There is no ptyalin in the saliva of the dog, the cat and the fox (6 individuals). The slight diastatic power of these animals is due to traces of blood and lymph diastases.

(2) Herbivora: The diastatic power of the parotid and mixed saliva of monkeys (7 individuals) is the same or less than that of man. The ptyalin concentration in the rabbit's parotid saliva is the same or slightly greater than that of man. But the parotid and mixed saliva of the goat (6 individuals) and the horse (14 individuals) have no diastatic power.

(3) Thus, while the absence of ptyalin in the saliva of many (probably all carnivora) and its presence in rodents and primates

may suggest adaptation, the absence of it in some herbivora nullifies such a conclusion. The saliva of monkeys ought on the adoption hypothesis to have greater ptyalin concentration than that of man. But we do not wish to be understood as holding that the ptyalin producing processes have been evolved without any relation to the nature of the food, because we must have data from all the mammalian groups before we are in position to determine whether the absence of ptyalin signifies atrophy or incipient evolution.

II. *The Relation of Ptyalin Concentration to the Rate of Secretion of the Saliva.*

(1) Weak acids (acetic) in the mouth is a more efficient stimulus to the secretion of the parotid than mechanical stimuli (dry sand, crackers, flour, cotton) and within limits the stronger the acid the greater the rate of secretion. The concentration of the human parotid saliva varies directly with the rate of secretion, just as in the case of lower mammals.

(2) The concentration of the ptylin in the parotid saliva of the rabbit varies directly with that of the organic solids in the case of gland anemia and on stimulation of the cervical sympathetic nerve (Carlson and Ryan). Since in the rested gland the organic solids increase with the rate of secretion we would expect the rapidly secreted saliva to contain the highest percentage of ptyalin. This is the case in the individuals (man) who respond readily with varying secretion rates to stimuli of varying strength (different strength of acids, or sand and acids). Thus the slowly secreted saliva obtained on placing sand in the mouth contains less ptyalin than that secured on stimulation with acid. But this direct relation between ptylin concentration and secretion rate is not a close one, hence a great difference in secretion rate is required in order to demonstrate the difference in diastatic power. But this is also true of the organic solids. We have not yet been able to demonstrate this relation in the case of the rabbit's parotid saliva, probably because of the rapid fatigue of the gland under experimental conditions.

(3) Qualitatively different stimuli (acid, salt, sweet, bitter, mechanical, agreeable, disagreeable) yield no constant difference in the ptyalin concentration of the parotid saliva in man. But

these data are not conclusive owing to the practical impossibility of keeping the secretion rate uniform.

(4) In varying directly with the organic solids and the secretion rate it seems that the processes of ptyalin secretion differ from the ferment secreting processes in the other digestive glands.

Powlow's findings that in the dog dry sand in the mouth causes a rapid secretion of a very dilute saliva seem not to apply to man. In man the secretion rate varies directly with the strength of the stimulus in the mouth and the saliva concentration depends —within limits— on the secretion rate. There may be some difference in different mammalian groups as regards the efficacy of the different reflex stimuli in the mouth, as acids in the mouth do not produce a copious salivary flow from the rabbit's parotid.

Unless the factor of secretion rate is controlled in all work on saliva concentration and ptyalin concentration under different reflex stimuli and dietary conditions, the results obtained are not conclusive.

THE ACTION OF ISOTONIC SOLUTIONS OF NEUTRAL SALTS ON UNFERTILIZED ECHINODERM EGGS.

By RALPH S. LILLIE.

(From the Physiological Laboratory, Marine Biological Laboratory, Woods Hole, Mass.)

Unfertilized eggs of Arbacia placed in pure solutions of various neutral salts of the alkali metals isotonic with sea-water, show, after varying intervals, diffusion of pigment into the medium and eventually disintegration. The salts of the same metal differ in their rate of action; the order of relative effectiveness for sodium salts with monovalent anions is: $NaCl < NaBr < NaNO_3 < NaCNS < NaI$. Potassium salts have a more gradual effect of the same kind and show the same general order of relative activity. The loss of pigment is an effect analogous to hemolysis and indicates an increase in the permeability of the plasma membrane, due probably to change in the aggregation state of its colloids.

If eggs, after a relatively brief exposure (five to twenty minutes) to these solutions, are transferred to normal sea-water, a certain

proportion—small in solutions of chloride or bromide, but considerable or large in solutions of nitrate, sulphocyanate, and iodide—exhibit membrane-formation, irregular form-change, and cleavage, and a small proportion may develop to the blastula stage. The order of relative favorability for the different salts is the same as above. This agreement confirms the view that the primary change in the initiation of cell-division and development is an increase in the permeability of the plasma membrane.

The above order is also that of relative toxicity. The toxic effect is to be attributed mainly to the loss of diffusible cell-constituents through the altered plasma membrane; the exit of pigment is a visible instance of this kind. The irreversibility of the death-process—as contrasted (e.g.) with the reversibility of anesthesia where the normal permeability appears to be *decreased* rather than increased—depends on the chemical disorganization of the protoplasm resulting from extensive loss of its diffusible constituents through the now permeable plasma membrane.

THE FOOD REQUIREMENTS FOR GROWING CHILDREN.

By ELBERT W. ROCKWOOD.

(From the Chemical Laboratory, University of Iowa.)

The food used during 29 days by two boys, twelve and one-half and eight and one-half years of age, was determined. The quality and quantity did not differ from that usually eaten but all overeating was discouraged and, as far as possible, prevented. The amounts indicate that the usual standards for such children are too low. Fifty-nine calories and 75 calories per kilo of body weight were used by the twelve and one-half and the eight and one-half year old boy respectively.

THE SENSITIZING AND DESENSITIZING ACTION OF VARIOUS ELECTROLYTES ON MUSCLE AND NERVE.

By RALPH S. LILLIE.

(From the Physiological Laboratory, Department of Zoölogy, University of Pennsylvania.)

When a frog's gastrocnemius is transferred from Ringer solution to an isotonic($\frac{}{8}$)solution of NaI the muscle shows an imme-

diate increase in tone and usually begins a slight rhythmical twitching which persists during its stay in the solution. On return to Ringer solution prompt relaxation follows and the twitching ceases. If then the muscle is exposed for a brief period, e.g., three minutes, to a pure isotonic ($\frac{M}{8}$) NaCl solution and is again brought into $\frac{M}{8}$ NaI solution, the response is found to be markedly altered; the muscle shows a greatly increased rise of tone with vigorous twitching or incomplete tetanus. Other sodium salts show similar action; also lithium salts to a less degree. The effect varies with the nature of the anion: bromide and sulphocyanate are in general more effective than nitrate, chloride and chlorate. In $\frac{M}{8}$ Na acetate, sulphate and tartrate the muscle shows active twitching from the first and the response to $\frac{M}{8}$ NaI is decidedly greater than with the first named salts. On the other hand, exposure for three minutes to $\frac{M}{8}$ MgCl, $MgSO_4$, $CaCl_2$, or $SrCl_2$ greatly diminishes or altogether suppresses the response to $\frac{M}{8}$ NaI; $\frac{M}{8}$ $BaCl_2$ on the contrary has a characteristic sensitizing action. Weak solutions of acid ($\frac{M}{500}$-$\frac{M}{1000}$ HCl) in Ringer solution have marked desensitizing action; on the other hand alkali (KOH) in similar concentrations increases the height of the response.

With nerve essentially similar conditions have been found, although to produce a decided sensitization longer immersion in the solutions is usually required.

The variation in the above effects with the nature of the anion indicates a colloid action as their common basis. Since the salts penetrate the living cell very slowly if at all, their point of action must be superficial, i.e., the plasma membrane. Presumably they act by altering the colloids of the latter and so affecting its permeability; the readiness with which the sudden increase in permeability associated with stimulation is effected is thus increased or decreased.

THE ABSORPTION OF FAT STAINED BY SUDAN III.

By R. H. WHITEHEAD (by invitation).

(From the Laboratory of Anatomy, University of Virginia.)

This paper is presented in order to clear up certain points in the account of an experiment undertaken as a demonstration of

the common belief that fat is not absorbed unsplit[1], which account seems to have been open to misapprehension.

(1) I was fully aware that Sudan III is absorbed, having seen Dr. Gage's striking preparations at the Baltimore meeting of the Association of American Anatomists. But his paper did not appear until after mine was in press, and thus I could only refer to the meeting. However, the very fact that this dye is absorbed was my reason for employing it. For, I reasoned, if an absorbable dye so freely soluble in fat be introduced with fat into the intestinal canal, the absence of stained granules in the villi would constitute fair proof that fat is not absorbed unsplit. But I had no interest at the time in the absorption of Sudan III *per se*, except in so far as it might, or might not, be carried in by unsplit fat. As a matter of fact, microscopical examination of frozen sections of the intestin revealed no red globules in the epithelium and lacteals of the villi. That, however, fat had been absorbed in some form could be demonstrated by subsequent staining of the sections with Sudan III.

(2) The failure to observe pink material in the lymphatics of the mesentery may have been due, as has been suggested, to post mortem contraction of those vessels. At the time I supposed that it was accounted for by the early stage of digestion at which the examination was made. A subsequent experiment upon a living cat leads me to believe that it was due both to the early stage of digestion and to the small quantity of material present: a *small* quantity of fat given to a *fasting* animal produced so little chyle that the vessels were not distinct.

(3) My remarks as to the form in which the fat was actually absorbed in the experiment were merely an attempt to bring the findings into accord with the current views of physiologists. I knew that there was reason to believe that fatty acids may be absorbed in solution; but it seemed that the fact that the sections lost their pink color in 80 per cent alcohol and the further fact that a soap made with oleic acid and sodium appeared to dissolve the dye very feebly were opposed to that form of absorption in the particular case, and that the presumption was in favor of soap. The point, however, seemed to me immaterial to what I was trying

[1] Whitehead: *Amer. Journ. of Physiol.*, xxiv, 294. 1909.

to demonstrate; for in either event, whether the fat was absorbed as fatty acid or as soap, it had been split. I have no desire to insist upon the presumption, but merely wish to bring out the main contention in my note, which was that the experiment afforded a demonstration, histological of course, that the fat was not absorbed in the form of unsplit, emulsified fat.

THE EFFECT OF INANITION AND OF VARIOUS DIETS UPON THE RESISTANCE OF ANIMALS TO CERTAIN POISONS.

By REID HUNT.

(From the Division of Pharmacology, Hygienic Laboratory, Bureau of Public Health and Marine-Hospital Service, Washington, D. C.)

Mice, rats, guinea pigs, and dogs were fed upon a great variety of ordinary foods (bread, milk, eggs, oatmeal, beef, ham, cheese, olive and other oils and fats, liver, dextrose, potatoes, rice, etc.) or upon various mixtures of these foods and after a few weeks their resistance to a number of poisons (morphine, cocaine, acetonitrile, etc.) determined. Marked variations in the resistance was noted; in extreme cases it required 30 times as large a dose of a definite poison to cause the death of mice fed upon one diet as of those which had received a different diet. These results cannot be due to the relative proportion of "protein," "fat," or "carbohydrates." They are evidently due to certain specific effects of the foods. In one case (the increased resistance to acetonitrile of mice fed on oatmeal) the cause could, with a high degree of probability, be traced to an effect of the diet upon an iodine-free constituent of the thyroid gland; it is believed that oatmeal contains some constituent which stimulates the growth or furnishes material for the growth of parts of this gland. It was suggested that the explanation of the value of an oatmeal diet in certain cases of diabetes might similarly be traced to a specific effect of this diet upon some of the hormones concerned in sugar metabolism and that further studies on these lines might throw light on the effect of diet upon the susceptibility of mice and other animals to carcinoma and various diseases.

The relation between rate of growth and resistance to certain poisons, the effect of partial inanition upon the latter, and a num-

ber of similar problems were discussed. It was shown for example that animals which had been kept upon a restricted diet were more (as much as three times as) resistant to acetonitrile as those which had been kept upon an unrestricted diet. By a study of the sulphocyanate excretion it was shown that this increased resistance was due to a diminished decomposition of the acetonitrile molecule by which a smaller amount of hydrocyanic acid, the poisonous agent, was produced. This is an illustration of how a definite metabolic activity may be lowered by partial inanition.

DO MUSCLE AND BLOOD SERUM CONTAIN KREATININ?

By P. A. SHAFFER and E. A. REINOSO.

(From the Laboratory of Chemical Pathology, Cornell University Medical School, New York.)

Using methods which it is believed allow no conversion of kreatin into kreatinin the authors find between 1 and 6 mg. of kreatinin per 100 gm. of fresh dog muscle, and about 1 mg. per 100 cc. of serum. This amount is roughly equivalent to the amount of kreatinin excreted in 24 hours (about 25 mg. per kilo of body weight or 50 mg. per kilo of muscle), and favors the view that the kreatinin excreted arises in muscle tissue.

On the reversible reaction for the liberation of carbonic acid from the blood in the lung. Lawrence J. Henderson.

The distribution of glycogenolytic ferment in the animal body. J. J. R. Macleod.

Metabolism of purin derivatives. Lafayette B. Mendel and John F. Lyman.

Third Meeting.

Thursday morning, December 30. Massachusetts Institute of Technology. Joint session with the Biological Section of the American Chemical Society.

Presiding officer: The chairman of the Biological Section of the American Chemical Society, Samuel C. Prescott.

THE SELECTIVE ANTISEPTIC ACTION OF COPPER SALTS.

By ALFRED SPRINGER.

(From the Laboratory of Dr. Alfred Springer, Cincinnati, Ohio.)

Last year I found a certain Cincinnati "Certified Milk" contaminated with traces of copper salt, which in some cases though containing only one part in two millions, decidedly affected the normal sequence of fermentative action and made the milk a better medium for the growth of certain moulds. In the course of my experimentation I found that the copper salts were highly selective, being most efficient in inhibiting the putrefactive germs, as evidenced by tests made with egg albumin, blood albumin, meat and other nitrogenous substances, with and without the addition of copper salts. These results can be caused either by the copper salts preserving the substances in their original condition, or splitting them without the formation of odorous compounds, or dissociating the odorous compounds themselves into non-odorous compounds.

It seemed to me that some light could be thrown upon the action of these salts by experimenting with copper treated eggs and then placing them in an incubator. In the first series of experiments I completely submerged many eggs in a cupric sulphate solution and check ones in distilled water. Those in the distilled water kept about two months, the others after a year's time have not become foul. When however eggs, which had been completely submerged several weeks in a copper or distilled water solution, were placed in an incubator no chickens hatched. The distilled water experiment showed that it is fatal to prevent air from reaching the germinative part of the egg. The preservative effects of the copper salts might have been due to their rendering the eggs to a condition similar to that of unfertilized ones (which keep far better than the fertilized) or they inhibited the putrefactive micro-organisms without affecting germinative properties. In order to determine this, I made another series of experiments by placing eggs upright in copper and distilled water solutions with the broad end projecting above the liquid so that air could enter into that part. In another set, one-half of the egg was longitudinally

immersed in copper sulphate solutions and distilled water twenty-four hours, then turned so that the other half would be immersed twenty-four hours, but at all times air had free access through the upper half. After 17 days' treatment, these eggs as well as some check untreated ones were placed in the incubator. On the twentieth day a chicken hatched from an egg which had been three quarters immersed in distilled water seventeen days. I waited five days longer no other chick coming out of any shell, I opened the eggs and found that two of the fertile ones had almost completely developed. One of these was from a partly submerged egg and the other from those which had been immersed and turned daily.

From the eggs containing the chicks, I sucked up part of the liquid with a pipette, digested it with sulphuric acid in a Kjeldahl flask and tested for copper. It was not even necessary to digest the liquid, as it could be diluted with water and electrolyzed direct, the copper depositing on the cathode. This evidently showed, as you see by these specimens, that embryonic growth to almost complete development took place, although the imbedded liquid was practically a copper bath. While these experiments are still very incomplete, it strikes me all signs point to the belief that small amounts of copper salts in their selective action towards the putrefactive ferments and their unpronounced effects on other enzymes, may be of great therapeutic value.

CONCURRENT OXIDIZING AND REDUCING POWER OF ROOTS.

By OSWALD SCHREINER and M. X. SULLIVAN.

(From the Laboratory of Soil Fertility Investigations, Bureau of Soils, Washington, D. C.)

The roots of growing plants, such as wheat, have the power to oxidize α-naphthylamine, benzidine, phenolphthalin, aloin, guaiac pyrogallol, etc. When indicators like α-naphthylamine and benzidine are used, the colors due to oxidation are most intense on the region of the root where growth is most active, the most marked oxidation showing by a distinct band of color just back of the root cap. Then comes a practically colorless zone and then a

colored zone, the color becoming less intense toward the upper part of the root. Wheat roots grown in sodium selenite solution neutralized by hydrochloric acid reduce the selenite with a pink deposit of selenium upon the root. This deposit is most marked a short distance back of the root cap just back of the region of greatest oxidative power and appears there first. The points of emergence of the secondary roots also show the color strongly. The reducing power is more active in the young and vigorous roots. Roots killed by being dipped in boiling water have no reducing action. Roots in the alkaline sodium selenite have little if any reducing action. In the main, with increased oxidizing power there is an increased reducing power. Potassium iodide in certain concentrations, however, retards oxidation but does not affect the reducing power and may even increase it.

RATIO OF PLANT NUTRIENTS AS AFFECTED BY HARMFUL SOIL COMPOUNDS.

By OSWALD SCHREINER and J. J. SKINNER.

(From the Laboratory of Soil Fertility Investigations, Bureau of Soils, Washington, D. C.)

Results of a comprehensive study of culture solutions with and without dihydroxystearic acid, a harmful compound isolated from soils, were reported. Sixty-six culture solutions comprised all possible ratios of the three principal fertilizer elements: phosphate, nitrate and potassium varying in 10 per cent stages were used in each series. The culture solutions were changed every three days and analyzed, the remaining composition and ratio of the above fertilizer elements being thus determined. In this way the effect of the plant and of the dihydroxystearic acid on the composition and ratio could be determined. The triangular diagram is used in this work and makes possible the intelligent handling and presentation of the results.

Some of the principal results were as follows: The plant growth and absorption were greatest in the solutions containing all three fertilizer elements, but not in equal proportions, the greatest growth and greatest absorption being found in the region below the center in the triangle. The dihydroxystearic acid had the

effect of shifting this region of greatest growth toward those ratios higher in nitrogen. Although absorption was greatest in this region, the ratios suffered the least change; the greatest change is produced in those ratios removed from this normal region.

The harmful soil compound greatly inhibited growth in all the solutions, but was the most harmful in those ratios not well suited for plant growth and least in those best suited for plant growth. Moreover, it is less harmful in the presence of those ratios mainly nitrogenous than in those mainly phosphatic or potassic and this effect is also associated with a higher nitrogen removal. The quantity of phosphate and potash removed was less in the presence of this compound. The investigations tend to throw much light upon the relations between plant growth, absorption, fertilizer action and influence of organic compounds.

A METHOD FOR THE DETERMINATION OF AMINO NITROGEN, AND ITS APPLICATIONS.

By DONALD D. VAN SLYKE.

(From the Laboratory of the Rockefeller Institute for Medical Research.)

Aliphatic amines, as is well known, react with HNO_2 according to the equation $RNH_2 + HNO_2 = ROH + H_2O + N_2$, and several methods have been devised for estimating NH_2 groups by measuring the nitrogen gas evolved by this reaction.[1] None of them has been sufficiently simple or accurate to attain general use, however. In the method proposed the nitrogen is freed in an atmosphere of pure NO, formed by spontaneous decomposition of HNO_2. The NO is afterwards absorbed by alkaline $KMnO_4$ solution, and the pure N_2 measured in a gas burette.[2] The operation requires but simple apparatus, a few minutes for completion, and is as accurate as a Dumas determination. Alanin, valin, leucin, glycin, aspartic acid, glutaminic acid, phenylalanin, serin, oxyprolin, tyrosin, arginin, histidin, tryptophan, leucyl-

[1] Meyer: *Anleitung zur quant. Best. d. organischen Atomgruppen.*

[2] Details of apparatus and manipulation are described in the *Proceedings of the Soc. for Exp. Biol. and Med.*, December 15, 1909. The apparatus can be obtained from Eimer and Amend, New York.

leucin, leucyl-glycin, cytosin, and guanin yield quantitatively one molecule each of N_2. Lysin yields two molecules of N_2. Prolin and glycin anhydride, which contain only imino N, do not react at all. Guanidin and its derivative, creatin, also fail to react, and the same is true of the acid amide N of asparagin. The latter gives off only one atom of N. Glycyl peptides evolve somewhat more than the theoretical one molecule of N_2, because the glycolyl radical, formed by the reaction of the glycyl NH_2, is unstable in the reaction mixture, and, being gradually broken off from the rest of the molecule, exposes the N of the second amino acid radical to the attack of HNO_2. The radicals formed from the other amino acids, except serin which behaves like glycin, appear stable under the conditions employed.

The method will be of value for convenient analysis in identifying the amino acids. It will serve also for estimating the amino N of substances of undetermined constitution, and of mixtures such as hydrolyzed protein.

In this connection it can be used to determine the degree of completeness with which a protein is hydrolyzed by acids, alkali, or enzymes. Native proteins show very little amino N, the N being nearly all bound undoubtedly in –CO–NH– linkings. As the protein molecule is broken down, the NH_2 groups are freed, and consequently amino determination affords a genuine chemical measure of the degree to which a digestion or hydrolysis has progressed.

It further renders possible an accurate determination of the prolin obtained by the ester method. The alcoholic extract of the amino acids whose esters distil below 100° contains prolin with more or less of the other acids as impurities. The amount of the latter can be determined by amino N estimation, and this N subtracted from the total, gives the prolin N.

Histidin and arginin, as obtained in solution by the Kossel and Patten method, can be identified without isolation. The ratio total N: amino N, in the case of histidin is 1:3, of arginin, 1:4, and as these ratios are characteristic, amino and Kjeldahl determinations on their separate solutions are sufficient to identify these bases.

The method has been made the basis of a quantitative determination of amino N (amino acids) in the urine. Urea and am-

monia react slowly with HNO_2, so must be removed. Seventy-five cc. of urine plus 2.5 cc. of concentrated H_2SO_4 are heated in an autoclave for 1½ hours at 175°. The urea is entirely changed to ammonia, which is boiled off after adding 10 g. of CaO. The filtrate from the $CaSO_4$, $Ca(OH)_2$. and other insoluble Ca salts is concentrated, diluted to 25 cc., and amino N determinations made on 10 cc. portions. Hippuric acid N may be determined separately by extracting an acidified urine with ethyl acetate, heating the extract with HCl and determining the amino N of the glycin set free. By the autoclave treatment, the amino N groups combined in the form of hippuric acid, peptides, etc., are freed and all determined. Determinations on a number of normal urines indicate a normal amino N of 2.0 per cent + 0.5 per cent of the total N. Urines from cases of muscular dystrophy, multiple scleroses, paralysis agitans, and cancer of antrum showed normal amino N content. The one case of gout showed 3.87 per cent. Of 8 cases of arthritis, 2 were normal (2 to 2.5 per cent), 5 between 3 and 5 per cent, and one very high, 5.56 per cent. The study will be continued to cover other conditions, including those of febrile and liver cases.

MANGANESE OF THE FRESH WATER MUSSELS.

By HAROLD C. BRADLEY.

(From the Laboratory of Physiology, University of Wisconsin.)

It is believed that manganese is a normal tissue element of the Unionidæ fresh water mussels. Many hundred specimens have been examined during the last four years and the element has in every case been present abundantly in the specimens. The mussels examined represent practically all the important drainage systems of the United States and Canada east of the Rocky Mountains. The ability to migrate rapidly and to cross from one watershed to another while parasitic in the gills of fishes makes it all the more probable that the specimens collected actually represent the entire family throughout the country. Manganese can readily be demonstrated in the blood, muscles, reproductive glands, nephridial organs, hepato-pancreas, gills and mantle of a single specimen. The latter two tissues contain it in highest

percentages. The eggs and embryos contain the element, and it is built into the nacre of the shell as well. It has been readily demonstrated in fossil shells of the same family where the nacre was well preserved. During starvation the element is strongly retained by the mussels; during the first few days the excreta will be found to contain the element in decreasing amounts; finally the feces become quite free from manganese. Other metallic elements are excreted continuously during starvation, so that the tissues grow poor in ash, but the ash becomes richer in manganese showing again a definite retention. The source of manganese is the food of the mussels—crenothrix and diatoms always found associated with them are able to concentrate manganese from its very dilute solution in the water where they live. It is noticeable that in lakes where the water is very pure and free from manganese neither crenothrix nor mussels are to be found, while in other lakes in direct communication, but whose water seeps through glacial drift and thus is richer in inorganic salts, both are present It is believed that the manganese serves a respiratory function in the tissues and blood of these lamellibranchs.

THE DETERMINATION OF ARSENIC IN ANIMAL TISSUE.

By CHARLES R. SANGER and WILLIS A. BOUGHTON.

(From the Chemical Laboratory of Harvard College.)

An extension of the method of Sanger and Black for the determination of arsenic in urine. The tissue is distilled with concentrated hydrochloric acid into concentrated nitric acid. The distillate is evaporated with sulphuric acid, and the arsenic determined by the Gutzeit or Marsh method.

THE ESTIMATION OF MORPHINE IN CASES OF POISONING.

By CHARLES R. SANGER and WILLIS A. BOUGHTON.

(From the Chemical Laboratory of Harvard College.)

The well known test of Kobert and others is made approximately quantitative. The morphine residue is treated with a

few drops of a solution of 2 cc. of formaldehyde (40 per cent) in 60 cc. of concentrated sulphuric acid. After standing, the blue residue is diluted, neutralized with sodium hydroxid, and the resulting brown solution is made up to a definite volume in a Nessler tube. Comparison standards are made by treating known amounts of morphine in a similar way.

THE PHOSPHORUS OF THE FLAT TURNIP.

By BURT L. HARTWELL and WILHELM B. QUANTZ.

(*From the Laboratory of Chemistry, Rhode Island Agricultural Experiment Station.*)

It was found that the percentage of phosphorus in the dry matter of flat turnips was influenced by the amount of available phosphorus in the soil upon which they were grown. This led to the attempt to ascertain if any particular class of the phosphorus compounds was influenced principally.

About 10 per cent of the phosphorus of the dry turnip was soluble in 95 per cent alcohol, and about 70 per cent was dissolved subsequently in 0.2 per cent hydrochloric acid. Fifty to 70 per cent of the phosphorus in this extract was precipitable by a molybdenum mixture containing only a small amount of free nitric acid. In fresh turnips about 80 per cent of the total phosphorus was found in the somewhat colloidal aqueous extract, and over four-fifths of this was directly precipitable by magnesium oxid and by the official mixtures of molybdenum and magnesium.

Nearly all of the phosphorus in turnip juice passed through a dialyzer. When added to a standard solution of sodium phosphate the colloidal matter left within the dialyzer interfered with the complete precipitation of phosphorus by the molybdic method. Hydrochloric acid added to turnip juice itself to the extent of 0.2 per cent made it possible after filtration to precipitate practically all of the phosphorus directly from the filtrate. Practically no phosphorus in phytin was present in the juice. It appears as if four-fifths of the phosphorus of fresh flat turnips is in soluble compounds and exists mainly as so-called inorganic phosphorus.

THE ACTION OF ENZYMES ON SUGARS.

By C. S. HUDSON.

(From the Laboratory of the Bureau of Chemistry, United States Department of Agriculture.)

The decompositions of sugars by enzymes can be accurately measured and the reactions are therefore most suitable for the study of enzyme action. A summary of recent work along this line shows that the laws of the action of invertase and emulsin are much more simple than earlier investigators had believed, the reason being that the earlier work is now known to be erroneous experimentally, because the marked effect of the mutarotation of the sugars was not considered.

THE CAUSE OF THE DIGESTION DEPRESSION PRODUCED BY MOLASSES.

By J. B. LINDSEY and P. H. SMITH.

(From the Laboratory of the Massachusetts Agricultural Experiment Station, Amherst.)

Our own numerous experiments,[1] as well as those of other investigators, have shown that molasses exerts a distinct digestion depression upon those feedstuffs with which it is fed. This depression appears to vary depending upon the character of the feed, the amount of molasses fed and the individuality and condition of the animal. The addition of considerable amounts of sugar and starch have been shown to produce similar results.[2]

It is claimed by some investigators that the depression is due to an increased peristalsis of the intestines, which causes a less complete digestion of the food. In order to gain additional light on this matter, a number of observations were made with sheep at this station using lampblack as an indicator. A summary of the results follows:

[1] In the twenty-second (forthcoming) report of the Massachusetts Agricultural Experiment Station.

[2] See the numerous experiments of Henneberg and Stohmann, Kühn and Fleischer, E. Wolff, etc., in the *Journal für Landw.* and in the *Landw. Versuchs.*

SHEEP NUMBER	NORMAL RATION. LAMPBLACK APPEARED IN (HOURS)	MOLASSES RATION. LAMPBLACK APPEARED IN (HOURS)	NORMAL RATION. LAMPBLACK DISAPPEARED (HOURS)	MOLASSES RATION. LAMPBLACK DISAPPEARED (HOURS)
II	20			
I	19		139	
II	15.5		144.	
IV		22		
III		21		96
IV		14		134
III		22		144
Average	18.2	19.7	141.5	139

It is evident that these results do not show sufficient variation to warrant a conclusion that the molasses exerted any peristaltic action. It is to be admitted that the lampblack did not prove as sharp an indicator as was desired. It is intended to make additional observations of a similar character using another indicator and also to continue our inquiry relative to the cause of the .epression.

CHEMICAL ORGANIZATION OF A TYPICAL FRUIT.

By A. E. VINSON.

(*From the Chemical Laboratory of the Arizona Agricultural Experiment Station.*)

It was shown that unripe dates of the invert sugar type contained high percentages of cane sugar which they retained so long as the fruit remained intact. Juice collected at the mill and filtered at once to remove pulp was high in cane sugar. Successive fractions obtained under the Buchner press showed that very rapid inversion was taking place in the presence of the pulp. All fractions after inversion with HCl gave substantially the same polarizations. The filtered juice contained no invertase excepting a trace in the portion obtained under low pressure which was somewhat turbid. The nitrogen in the juice increased with increasing pressure. So long as mechanical integrity of the fruit is retained contact between cane sugar and invertase is reduced to the min-

imum. When the tissues are crushed free access of the soluble cane sugar to intracellular invertase is established, due to removal of mechanical obstructions. The intracellular invertase, however, remains permanently with the pulp, although soluble nitrogenous cell constituents escape more and more freely with increasing pressure.

FIXING AND STAINING TANNIN IN PLANT TISSUES.

By A. E. VINSON.

(From the Chemical Laboratory of the Arizona Agricultural Experiment Station.)

The content of tannin cells in dates and persimmons is deposited and stained brown by the vapor of ethyl or amyl nitrite. Only those cells which have been shown to contain tannin by other reactions are stained. A Japanese persimmon weighing one half pound was stained entirely through in about twenty-four hours. The method is neat, simple and avoids all danger of dragging soluble tanning into neighboring cells since the specimen is stained before being cut. Various plant organs show marked differentiation of tissues after treatment with the vapor of nitrous ether, and isolated tannin cells are easily located in the hand specimen. The juice of unripe date *in vitro* is precipitated suddenly after the lapse of several moments. The precipitation is prevented by alcohol or glycerol. Persimmon juice is colored deep wine-red but is not precipitated by nitrous ether.

For laboratory use a twenty per cent solution of commercial nitrous ether in alcohol is recommended.

DESTRUCTION OF INVERTASE BY ACIDS AND ALKALIES.

By H. S. PAINE.

(From the Laboratory of Animal Physiological Chemistry, Bureau of Chemistry, U. S. Dept. of Agriculture.)

Samples of the same invertase preparation were kept at a constant temperature of 30 degrees for different time intervals in acid (HCl) and alkaline (NaOH) solutions at different concen-

trations. At the end of the respective time periods all the samples were brought to the same acidity (the acidity favorable to maximum activity of the enzyme) in cane sugar solutions of the same strength, all volumes being equal. After an inverting action of one hour, the velocity coefficient, k, of the rate of the inversion was calculated from the formula for monomolecular reactions, viz: $k = 1/t \log (R_0 - R_\infty / R - R_\infty)$, where R_0 is the rotation of the pure cane sugar solution, R_∞ the rotation of the same solution after complete inversion, and R the polarization at the time t, seconds and decimal logarithms being used in the calculation. The activity of the enzyme, as measured by the above coefficient, k, was found to decrease as the strength of the destroying acid or alkali solution was increased.

By an application of the above formula a coefficient, k_2, measuring the rate of destruction of the invertase was obtained as a derived value of the coefficient just referred to.

Destruction commenced at about 0.015 normal in acid and 0.01 normal in alkaline solution, requiring about five to six hours for completion at those concentrations. It was very rapid and required only about five minutes in 0.05 normal acid and 0.04 normal alkaline solution, showing that, while invertase is inactivated in very faintly alkaline solutions, the destructive action of alkalies on it is not much greater than that of acids.

In view of the fact that the degree of acidity or alkalinity of the media in which many enzymes naturally occur is subject to change, quite often between wide limits, investigations, such as the one just described, are of value in determining just when inactivation or destruction takes place. The contents of the alimentary tract of the higher animals (considered in its entirety). may be mentioned as an example of such a medium in which variations in acidity and alkalinity occur.

CORNIN, THE BITTER PRINCIPLE OF CORNUS FLORIDA.

By EMERSON R. MILLER.

(*From the Laboratory of Pharmaceutical Chemistry, Alabama Polytechnic Institute, Auburn, Alabama.*)

The root bark gives the best yield. Carpenter considered the bitter principle to be an organic base. The compound separated

by Geiger had a slight acid reaction. In pure condition it is perfectly white, has neither basic nor acid properties, is extremely bitter and crystallizes in fine silky needles or beautiful rectangular plates, according to conditions. Melting point: 181°C.

The substance is readily soluble in water and sparingly soluble in cold alcohol or cold acetone, but is dissolved to a considerable extent by these liquids at the boiling temperature. It is almost insoluble in ether, chloroform, benzole, petroleum ether and acetic ether, but is sparingly soluble in benzole or acetic ether at the boiling temperature. Contrary to Geiger's statement, its aqueous solution does not form a precipitate with either silver nitrate or lead subacetate.

Tested for nitrogen with soda-lime or metallic potassium, it gave negative results.

An aqueous solution, after standing some time assumes color and reduces Fehling solution. After heating with a little alkali or acid it reduces Fehling solution at once. It also reduces ammoniacal solutions of silver nitrate and bismuth subnitrate in the presence of alkali, and responds to Pettenkofer's test for glucose. An aqueous solution does not form a precipitate with phenylhydrazine hydrochloride, but on heating yields a yellowish red precipitate.

The average of ten analyses gave C = 52.49 per cent; H = 6.17 per cent. Computed for the formula $C_{17}H_{24}O_{10}$, C = 52.57 per cent; H = 6.18 per cent. A molecular weight determination by the freezing point method gave 377. The above formula requires 388. The average of two tests for methoxyl gave 7.48 per cent. One OCH_3 requires 7.98 per cent.

Cornin thus appears to be a glucoside whose molecule contains the glucose nucleus and, so far as determined, is represented by the formula $C_{16}H_{21}(OCH_3)O_9$.

Chairman's address: Industrial bacteriology as a field for biochemical investigation. Samuel C. Prescott.

The anaphylactic reaction as a specific test for protein. M. J. Rosenau.

Stagnation vs. circulation in house air. Ellen H. Richards and Royce W. Gilbert.

The relation of typhoid fever to the water supplies of Illinois. Edward Bartow.

Studies on the physiological and chemical toxicology of the sap of the manzanillo tree. Jose A. Fernandez Benitez.

The starting point in the bacterial decomposition of the flesh of dressed fowls. Mary E. Pennington and E. L. St. John.

Fourth meeting

Thursday afternoon, December 30. Harvard Medical School.
Presiding officer: The president, Otto Folin.

THE NUTRITIVE VALUE OF SOME SOLUBLE PENTOSANS, MANNANS, LEVULANS AND GALACTANS.

By MARY DAVIES SWARTZ. (By invitation.)

(*From the Laboratory of Physiological Chemistry of the Sheffield Scientific School in Yale University.*)

Investigations as to the fate in the animal body of certain water-soluble hemicelluloses, derived from marine algæ and related substances, show that they are very resistant to the action of animal and vegetable enzymes, such as human saliva, malt diastase, "Taka" diastase, pancreatic juice of dogs; extracts of pig's pancreas, of pig's stomach or of dog's intestines.

Experiments with the ordinary aerobic bacteria of the alimentary tract; with mixtures of soil and fecal aërobes, of soil and fecal anaerobes, and of powerful putrefactive organsims such as *B. anthracis symptomatici* and *B. maligni œdematis*, show that galactans are not appreciably affected by any of these. Pantosans, mannans, and levulans are gradually decomposed by soil and fecal bacteria, and by putrefactive anaerobes, sometimes with the formation of reducing substances.

When introduced parenterally, either subcutaneously or intravenously, they are not retained or altered by the organism, but are gradually excreted in the urine. Feeding experiments on dogs and human subjects show that those hemicelluloses most readily attacked by bacteria disappear most completely from the alimentary tract. Galactans, which are unaffected to any appreciable extent, are excreted in amounts averaging 75 per cent;

pentosans and mannans, hydrolyzed by bacteria, disappear almost entirely during the processes of digestion.

It is manifestly impossible to treat of the digestibility of hemicelluloses as a class; each group must be studied separately, and distinctions made also between soluble and insoluble forms.

The experiments give little justification for considering these carbohydrates as typical nutrients for man.

ON THE DETERIORATION OF DIPHTHERIA ANTITOXIN.

By EDWIN J. BANZHAF.

(From the Research Laboratory of the Department of Health, New York City.)

Lots, in duplicate, of native antitoxic sera, antitoxic citrated plasma and concentrated antitoxin globulin solution were taken. One lot of each was kept at ice-box temperature, varying from 4° to 7° C. The remaining lots at room temperature (22° to 26° C.)

On starting this work, it was my intention to retest these lots every two months, but after several retests, found it was too expensive an undertaking. I, therefore, lengthened the retest time to six months and later to once a year. The unit value of these lots of serums was determined very carefully with a toxin that was standardized every two months against a standard test serum furnished by the Hygienic Laboratory of the Public Health and Marine Hospital Service.

The deteriorations of these various lots were as follows:

The native antitoxic sera kept in ice-box: the average deterioration for one year was 14 per cent; for two years the average was 22 per cent; for three years 24 per cent; and for four years 25 per cent.

For the antitoxic citrated plasma, kept in an ice-box, the deterioration was extremely low. The average for one year was 6 per cent; for two years 8 per cent; for three years 9 per cent; and for four years 10 per cent.

The deterioration of potency with the concentrated antitoxic globulin solution, kept in an ice box for one year, was 13 per cent; for two years 17 per cent; for three years 20 per cent; and for four years 22 per cent.

The average deterioration of potency of the native antitoxic sera, kept at room temperature for one year, was 18 per cent; for two years 25 per cent; for three years 26 per cent; and for four years 29 per cent.

For the antitoxic citrated plasma, kept at room temperature, the average deterioration for one year was 8 per cent; for two years 10 per cent; for three years 12 per cent.

For the antitoxic globulin solution, kept at room temperature, for one year, the average deterioration was 16 per cent; for two years 20 per cent; for three years 23 per cent; and for four years 28 per cent.

A STUDY IN REPEATED FASTING.

By PAUL E. HOWE and P. B. HAWK.

(From the Laboratory of Physiological Chemistry of the University of Illinois.)

A fox terrier bitch about one year old and weighing 3.41 kg. was subjected to two fasts. On the fifteenth day of the first fast the premortal rise in nitrogen output was noted and was accompanied by other signs indicating that death would result in a few hours. She was carefully fed and during a feeding period of 47 days regained her former weight and was again brought into nitrogen equilibrium, after which she was fasted a second time. The water ingestion was uniform (250 cc.) throughout the feeding and fasting periods.

The first fast was 15 days in length and was marked by a rapid decrease in weight, a large increase in nitrogen excretion, followed by a pronounced premortal rise; a slight increase in ammonia, a gradual fall in creatinin, a drop in creatin below the creatinin value, followed by a pronounced rise, a few days before the premortal rise.

The second fast was twice as long as the first, i.e., 30 days, and was accompanied by a more gradual loss in weight, a much lower and fairly constant nitrogen excretion, a slight rise in ammonia, a less pronounced fall in creatinin, and a drop in the creatin more gradual than that of the first fast, but also accompanied by a rise toward the end.

The total output of creatinin for the second fast was about 60 per cent greater than that for the first fast, whereas the crea-

tin output was approximately the same in each instance. The output of allantoin and purin nitrogen was decreased during each fast from that observed during the feeding periods.

The loss in body weight was 45.8 per cent in the first fast and 46.8 per cent in the second fast. A table giving data regarding nitrogen distribution is appended.

Nitrogen excretion per day.
(*grams*)

	TOTAL N	UREA N	AMMONIA N	CREATININ N	CREATIN N	ALLANTOIN N	PURIN N	UNDETERMINED N
First fast (15 days)............	2.313	2.065	0.103	0.025	0.046	0.005	0.005	0.067
Second fast (30 days)..........	1.314	1.141	0.083	0.023	0.026	0.004	0.005	0.032

FASTING STUDIES ON MEN AND DOGS

By PAUL E. HOWE, H. A. MATTILL AND P. B. HAWK.

(*From the Laboratory of Physiological Chemistry of the University of Illinois.*)

The experiments reported were made upon two men and three male dogs. The dogs were first brought into nitrogen equilibrium then subjected to fasting, uniform volumes of water being given daily by means of a stomach tube. Of the men, one had subsisted for a period of over six months upon a mixed diet yielding not over forty grams of protein per day, whereas the other subject had been living for a similar period on a "meat free" diet yielding about 60 grams of protein per day. These men were placed on a uniform diet for a period of 7 days before fasting. During the fast their water ingestion was uniform from day to day. The daily loss in body weight was recorded for all subjects.

Dog No. 2 (adult) fasted 48 days losing 52.9 per cent of body weight and dog No. 6 (pup, one month old) fasted 6 days, losing 22.2 per cent of body weight. With dog No. 3 (adult) we have recorded the longest fast on record, i. e., 117 days, the next longest fast being one of 98 days reported by Kumagawa. Upon the 117th day, and showing a loss of 63 per cent of body weight. the

dog was carefully fed and brought back to nitrogen equilibrium and started on a second fast. The men each fasted 7 days and lost 7.7 per cent of body weight respectively.

Total nitrogen and urea in the cases of the adult dogs underwent an irregular fall from the beginning of the fast to the premortal rise, whereas in the case of the pup they rose quickly during the first part of the fast then fell more gradually to the inception of the premortal rise. The percentage of total nitrogen as urea in the case of the pup underwent a gradual fall from 90.8 per cent on the first day of the fast to 85.2 per cent on the last day. This relation was not noted in the case of adult dogs, the tendency here being for the percentage output of the urea to increase during the last portion of the fast. The former relation was again in force with the fasting men. The ammonia excretion of the pup increased daily from the beginning to the end of the fast, the ammonia excretion of the human subjects being somewhat similar. This regularity was not observed in connection with adult dogs.

The creatin excretion of the adult dogs fell during the first part of the fast and from the minimum level, assumed about midway in the fast, there was a gradual increase to the end. In the case of the pup the creatin rose from the first day of the fast, decreasing slightly just before the premortal rise. Creatin was also present in the urine of fasting men, in one case the rise being abrupt and in the other case gradual. Creatin in the case of adult dogs and men, fell in general gradually during the fast, whereas the creatinin excretion of the pup reached its maximum midway in the fast. In the case of those dogs which were fasted to the premortal rise the creatin curve rose above that of creatinin the point of crossing being earlier the younger the subject. There was a rather definite relationship in all our fasting experiments (exclusive of repeated fasts) between the crossing of the creatin and creatinin curves and the fall in nitrogen excretion, preceding the premortal rise, the crossing occurring from two to three days before the beginning of the fall. This crossing was not observed with the dog that fasted 117 days, nor with the men. The average daily creatin excretion was only about one-fourth as great during the 117 day fast as during the 6 and 48 day fasts. The adult dogs excreted less allantoin and purin nitrogen during the fast than during the feeding period, whereas

these excretions in the case of the pup were both increased during fasting.

NOTE ON THE PROTEIN METABOLISM OF PARTURIENT WOMEN.

By J. R. MURLIN and THORNE M. CARPENTER.

(From the Physiological Laboratory of Cornell University Medical College, New York City and the Nutrition Laboratory of the Carnegie Institution of Washington, at Boston, Mass.)

Urines collected during the last two to four weeks of pregnancy and the puerperium from the three cases used by us in determining the energy metabolism[1] of the parturient period have been analyzed for the usual nitrogen and sulphur fractions. The patients had been kept on a fairly constant diet containing about 9 grams of protein and 28 to 30 calories per kilo of body weight, during the entire period of observation, except one or two days immediately following parturition when they were on the regulation milk and broth diets.

The results show the usual parallelism between the total nitrogen and total sulphur. Just previous to parturition a noticeable rise in the creatin excretion was observed on one patient who was on a purin-free diet and on one of the other patients who was on a diet containing a small quantity of meat. The creatin also was high during the puerperium owing to the resolution processes going on in the uterus, but returned to normal within two weeks in the one case on whom the observations were carried that far. The observations accord with those already made by Murlin on parturient dogs.

[1] *Proceedings of the Society for Experimental Biology and Medicine*, 1909, vii, p. 17.

CHEMICAL AND MYCOLOGICAL STUDIES UPON A CORN ROT HAVING POSSIBLE RELATION TO THE ETIOLOGY OF PELLAGRA.

By HOWARD S. REED.

(From the Laboratory of Mycology and Bacteriology, Virginia Polytechnic Institute, Blacksburg, Va.

The fungus *Diplodia zeae* which causes a dry rot of maize has become more prevalent in America in recent years. In view of the increasing spread of pellagra the study of Diplodia seemed worthy of attention.

Maize when infected with pure cultures of *D. zeæ* and fed to mice produced death in a few days. Chemical examination of the infected meal showed the presence of an alcohol soluble body resembling Lombroso's pellagrozein. Other bodies showing a crystalline structure have been isolated. The physiological effects of these bodies are being investigated.

SOME URINARY FINDINGS IN ECLAMPSIA.

By L. B. STOOKEY.

(From the Physiological Laboratory, Medical School, University of Southern California.)

Five eclamptic urines were studied. The most prominent findings seem to consist in low urea nitrogen (70 per cent to 83 *per cent of total nitrogen), high ammonia nitrogen* (5 *per* cent to 10 per cent of total nitrogen), and high mono-amino-acid nitrogen (2 per cent to 6 per cent of total nitrogen) and a positive para-dimethylaminobenzaldehyde reaction of a variable intensity. In all cases a low protein dietary accompanied by frequent high colonic lavage with saline containing 0.3 per cent sodium carbonate and 0.1 per cent lysol led to a change toward normal in the nitrogenous distribution, a decreased para-dimethylaminobenzaldehyde reaction and lessened clinical manifestations. In two cases in which the eclamptic seizures although of less severity continued, the administration of iodids and thyroid led to a marked improvement of the symptomatic manifestations, in the nitrogen distribution and in the para-dimethylaminobenzaldehyde reaction.

ON THE UTILIZATION OF THE AMINO-ACIDS AND POLYPEPTIDS BY THE TUBERCLE BACILLUS.

By W. F. KOELKER and B. W. HAMMER.

(*From the Organic and Hygienic Laboratories, University of Wisconsin.*)

Earlier investigators have found that nitrogenous organic substances or mixtures of such (albumoses, peptones, amino-acids, blood serum, etc.) can be used as sources of organic nitrogen in culture media for the tubercle bacillus. Of this variety of substances the amino-acids were the only ones of known structure and of these but few had been synthesized. During the last 15 years the chemistry of this important group of protein cleavage products has been revolutionised. New components of the protein molecule have been found and synthesized, the optically active forms have been prepared and the active as well as the inactive united to the so-called polypeptid.

After repeating and duplicating some of the work of Proskauer and Beck on mixtures of amino-acids, the amino-acids glycin, alanin, aminoisobutyric acid, valin and leucin were studied, and all of the possible inactive di- and some of the tri-peptids formed by combining two or three molecules of glycin, alanin and leucin. Substitution products of glycocol containing secondary and tertiary amino-nitrogen were also investigated, *e.g.*, sarcosin, betain and creatin. The culture media were composed of 0.6 cc. of Kuehne's ash mixture, 0.2 gram of the organic nitrogen compound and 50 cc. of water. Each compound was tested individually. The results obtained were postive in some cases and negative in others. There seems to be evidence of a difference in the biological behavior of the homologous and the methyl-substituted amino-fatty acids. Glycin and alanin are favorable and give abundant growths, while other members of the series, amino-isobutyric acid, valin and leucin give no growth. Leucin was used in the two active forms and also in the racemic forms. The results obtained with the substitution products of glycin were negative in the two series of experiments; the same was found in the case of the active forms of leucin. The action seems to depend upon the following factors:

(1) The structure of the individual amino-acids.

(2) The primary, secondary or tertiary nature of the amino group.

(3) The order of the different amino-acid radicles in the polypeptids.

The investigation is being continued with the active peptids. Attempts are being made to determine whether a desamidation occurs in the case of the amino-acids, and whether the polypeptids are desamidized and then hydrolized, or not hydrolized at all.

OBSERVATIONS ON CAFFEIN GLYCOSURIA.

By WILLIAM SALANT and G. W. KNIGHT.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington, D. C.)

Experiments carried out on rabbits show that caffein when given in sufficient quantity induces glycosuria, which usually appears within two or three hours after its administration and may last several hours, but not over twenty-four hours. Sugar has been found in the urine when caffein was injected subcutaneously or when it was given by mouth, although larger doses are required to produce glycosuria when caffein is introduced into the stomach. Glycosuria has been observed after administering caffein to rabbits which had been kept on an exclusive diet of carrots for several days before the experiment, as well as in animals which had been fed on oats alone. Smaller quantities of caffein were required, however to induce glycosuria when the diet consisted of carrots. When the rabbits were starved four or five days, then fed fifteen or twenty grams of cane sugar, the effective dose of caffein necessary to produce glycosuria was smaller than in rabbits kept on a diet of carrots. A large number of observations were made on the effect of calcium chlorid on caffein glycosuria. They indicated that much smaller quantities of caffein will produce glycosuria when 8 to 10 cc. of $\frac{M}{8}$ molecular solution of calcium chlorid per kilo were injected into the ear veins of rabbits before or immediately after caffein was injected. No sugar was found in the urine when calcium chlorid was given in the same way but without caffein. Experiments on cats likewise show that caffein may produce glycosuria. The amounts of sugar found in the urine of such cats

varied between 1.65 and 5 per cent. Glycosuria cannot be produced, however, in these animals with the same facility as in rabbits. No glycosuria has been observed in dogs kept on a diet containing a large amount of carbohydrates, after the administration of caffein, although many of experiments have been made.

NOTES ON SENSITIZATION WITH TUBERCULIN TO TUBERCULAR RABBIT-SERUM.

By JAMES P. ATKINSON and C. B. FITZPATRICK.

(From the Chemical and Research Laboratories, Department of Health, New York City.)

These notes present some of the results of our study of sera, toxins, and related substances. We have noted by means of the kymograph the changes in pressure and respiration produced when we injected into normal dogs, treated with tuberculin or other toxic substances, the sera of normal and diseased animals. A number of interesting facts were found in this way, regarding substances in the serum of animals having tuberculosis, which we think worth reporting.

The tubercular rabbit serum with which these results were obtained, was drawn from rabbits which had been infected 35 days previously with a virulent culture of bovine tuberculosis injected intravenously. The serum was defibrinated and separated centrifugally.

We obtained the described reactions with the serum both before and after filtration through a Berkfeld filter. The serum which was employed the day after drawing gave the reaction and serum, after standing in the ice-box for ten days, also gave it. The same serum after three weeks did not give the reaction.

Injection Experiments.

The anaesthetic used consisted of 10 mgms. of morphine sulphate followed in about 30 minutes by 1.5 gram of chloretone and a little ether. The animals used were dogs averaging 30 pounds in weight.

The results obtained are summarized as follows:

(1) Dogs injected intravenously with 3 cc. of serum from a rabbit infected with tuberculosis do not suffer a reduction in arterial pressure. The serum was injected into the femoral vein and pressure taken in the carotid. Protocol of Nov. 17, 1909.

(2) Dogs that have been injected intravenously with 5 cc. of crude tuberculin and five minutes later with 3 cc. of serum, (administered in the same way), which had been obtained from a tubercular rabbit do not suffer a reduction in arterial pressure. The same is true when the serum is administered first and then the tuberculin five minutes later. Protocol of Nov. 17, 1909.

(5) *Dogs sensitized by a subcutaneous injection of 5 cc. of tuberculin, which is followed in from 12 to 18 hours by an intravenous injection of 1.5 to 3 cc. of tubercular rabbit serum*, DO SUFFER A MARKED REDUCTION IN ARTERIAL PRESSURE.

(4) This reaction is specific as far as we have been able to test it. (Protocol of December 4, 1909.) The following sera have been tested; glanders (horse), typhoid (human), antityphoid (horse), antistreptococcus (horse), normal rabbit, normal horse and a 2 per cent solution of Witte's peptone. We have one (1) record showing that the five weeks old tubercular rabbit serum which did not cause the reaction in a sensitized dog, was reactivated by the addition of an equal amount of fresh rabbit serum.

(5) Based on this reaction an attempt was made to immunize two rabbits against tuberculosis but without success. The rabbits were injected with 1 cc. of the crude tuberculin and beginning with the next day received 2 cc. of the tuberculosis serum on alternate days during a period of 10 days. Each rabbit received five injections of 5 cc. each of the tuberculosis serum.

The subcutaneous injection of 10 cc. of the tuberculosis serum into three rabbits each of which had been previously sensitized by an injection of 1 cc. of crude tuberculin, caused death in from 24 to 50 hours.

(6) This reaction, if found to be sufficiently specific, may be used as another method for the diagnosis of tuberculosis. The test would be made on an animal sensitized with tuberculin, by an injection of the patients blood-serum.

(7) We have obtained in normal dogs, by the intravenous

injection of 4 cc. of serum from rabbits in the paralytic stage of hydrophobia (seventh day after subdural inoculation), a marked depression in the arterial blood pressure. A similar reaction was caused by the intravenous injection of a substance (probably cholin), in small doses, obtained from the brains of calves and rabbits.

(8) We made the following experiments, in order to ascertain what relation this reaction (No. 3, above) has to anaphylaxis, as we know it. Five dogs were employed. Dog No. 1 received a subcutaneous injection of 2 cc. of normal horse serum. No. 2 received 1-75th. No. 3 received $\frac{1}{100}$. No. 4 received $\frac{1}{1000}$. No. 5 received two injections of $\frac{1}{10000}$, with an interval of four days between the injections. None of these dogs yielded a reaction which could be recorded on the kymograph, when given intravenously on the following day, 10cc. of the same normal horse serum.

These results apparently indicate that the reaction is simply one of the many phases of sensitization or increased susceptibility, following the action of toxic substances. It resembles then the anaphylactic reaction only to the extent that, although differing from one another, each is simply one of the many phases of increased susceptibility. We have tentatively named the phenomenon observed by us, *vasophylaxis*.

THE PROPERTIES OF LINTNER SOLUBLE STARCH.

By E. D. CLARK (by invitation).

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

In a recently published method[1] for the determination of diastatic power by the saccharification of Lintner soluble starch, it was found necessary to correct for a seemingly discovered reducing power of the soluble starch itself. This observation made it desirable to study more carefully the properties of soluble starch used in such work. There has always been some doubt as to the exact nature and properties of soluble starch; some authors

[1] Meyer: "Determination of diastatic power, etc." Dissertation. Columbia University. 1908.

stating that it has reducing powers, others that it does not have this property. We prepared Lintner soluble starch according to Meyer's published directions and after long washing, etc., we at last obtained a product whose acidity, expressed in terms of $\frac{N}{100}$ NaOH, was as low as that of Meyer's best samples. Some of the potato starch used in the work was the commercial grade while the rest was made in the laboratory in the usual manner.

Dialysis experiments were conducted with different samples of soluble starch suspended in water, no heat being used to promote complete solution of the soluble starch. Such suspensions, when dialyzed in collodion bags against distilled water, gave dialysates showing a bright red color with iodine and in some cases reduction of Fehling solution. Two per cent solutions of soluble starch were made by adding the starch to water at 95° C. When these solutions were dialyzed as before, the dialysates showed about the same properties except that the coloration with iodine was more of a purple tint changing to blue with excess of iodine.

After such treatment, the soluble starch solutions in the bags after dialysis were poured into alcohol, this causing the formation of an opalescent colloidal solution. The starch did not settle on long standing but was precipitated immediately by the addition of *one drop* of 10 per cent NaCl solution. The soluble starch was dissolved and reprecipitated several times in this manner, and finally was tested with iodine and Fehling solutions. The former gave purple and blue colorations, the latter showed reduction in every case.

The clear alcoholic filtrate from each of these precipitates was carefully evaporated to dryness at 40° and taken up in a little water. These solutions showed reddish-brown colorations with iodine and strongly reduced Fehling solution. In all cases checks were made on all reagents used and also upon the alcohol, collodion, filter paper, etc.

We conclude that Lintner soluble starch carries associated with it, certain amounts of dextrins with reducing power, from which it can be only partially freed by dialysis or alcohol precipitation. It is probable, also, that the method by which commercial starch is prepared renders such starch less capable of yielding Lintner soluble starch of the least possible reducing power.

In connection with the study of Lintner soluble starch we pre-

pared some soluble starch of as low reducing power in the following way which is only a matter of minutes whereas the Lintner treatment cannot be completed in less than two weeks. A thick starch paste was made by pouring a suspension of 4 grams of potato starch (in 15 cc. of cold water) into 200 cc. of water at 95°. The paste was cooled to 40° and then 5 cc. of filtered saliva were added and the mixture rapidly stirred. In two or three minutes the whole was liquefied, when it was poured into one liter of 95 per cent alcohol. To precipitate the colloidal soluble starch in large flocks, a drop of 10 per cent NaCl solution was now added. The soluble starch was quickly filtered off and dropped into a little boiling water. The solution was immediately cooled in order to prevent hydrolysis. The ptyalin was completely destroyed in the process. The soluble starch was reprecipitated with alcohol. The soluble starch prepared by the digestive method is extremely soluble in water, which is not true of the Lintner product. The salivary protein contributed to it is probably a negligible factor in most experiments in which soluble starch may be employed.

ON THE PREPARATION AND PROPERTIES OF SOME ORGANIC PROTEIN COMPOUNDS.

By WALTER H. EDDY (by invitation).

(*From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.*)

Tendo-mucoid combines with strychnin to form compounds whose protein and strychnin characteristics are demonstrable even after rigorous methods of purification are employed. Tendomucoid combines with conin, piperidin, morphin and anilin to produce water-soluble and acid precipitable compounds. Caseinogen and yeast nucleoprotein are capable of combining with alkaloidal bases in the same manner as mucoid. Ovomucoid from hen's eggs and from shad roe shows similar properties, and forms with strychnin compounds which are neutral to litmus, soluble in water and give good protein color tests. These compounds show great lability and sensitiveness to environmental conditions similar to the conditions found in cells. They sug-

gest the possibility that such combinations actually occur in the living cells.

Histon prepared from thymus glands also combines with tendo-mucoid to form a *water-insoluble* compound which is soluble in 0.05 per cent Na_2CO_3 solution. This soluble compound is probably a soluble sodium-histo-mucoid compound. Histon and yeast nucleoprotein combine to form a compound with similar properties. Ovomucoid and histon combine to form compounds at least temporarily insoluble in water.

In all these cases the protein base, histon, acts in a manner exactly comparable to that of the alkaloidal bases. The compounds are evidently not so simple as inorganic salt compounds but comparable to them. They all suggest at least the possibility of duplicating in the laboratory intra-cellular combinations and promise to throw light on the synthesis of proteins in the cells.

FURTHER OBSERVATIONS ON AN IMPROVED METHOD FOR THE DETERMINATION OF THE AMMONIA NITROGEN IN URINE.

By MATTHEW STEEL.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

Further study of our improved method for the quantitative determination of urinary nitrogen confirms the conclusion published about a year ago.[1] The new method effects the liberation and collection of the entire theoretical yield of ammonia from urinary triple phosphate

ON THE EFFECTS AND FATE OF INJECTED CONNECTIVE TISSUE MUCOID.

By JACOB ROSENBLOOM AND WILLIAM J. GIES.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

Experiments on dogs with tendo-mucoid have given the following information:

[1] Steel: *Proc. Soc. Exp. Biol. and Med.*, vi, p. 127. 1909.

The *subcutaneous* injection of mucoid (in 0.5 per cent KOH), in amounts equal to 0.43, 0.56 or 0.59 gram per kilo, produced toxic symptoms for periods varying from 24 to 60 hours. The strengths of the injected solutions were 2.5 per cent, 8 per cent and 8 per cent respectively. Some of the toxic symptoms were twitching of various muscles, marked depression, drowsiness and restlessness. When 1.91, 1.95, or 4.37 grams per kilo (dissolved in 0.5 per cent KOH) were injected subcutaneously, marked toxic symptoms were elicited and death occurred within a period varying from 20 to 72 hours. The strengths of the injected solutions in these cases were 12 per cent, 10 per cent and 10 per cent respectively. Some of the toxic symptoms were extreme drowsiness, vomiting, shivering and restlessness as well as increase of pulse, temperature and respiration. The alkali introduced with the mucoid did not procure the toxic symptoms noted.

The *intraperitoneal* injection of 0.36, 0.69, 0.78 or 0.77 gram per kilo (dissolved in 0.5 per cent Na_2CO_3 or 0.5 per cent NaOH) produced peritonitis and death in 8 to 20 hours. Intraperitoneal injection of 0.69, 0.93 or 1.3 gram per kilo (dissolved in 0.5 per cent KOH) produced peritonitis and death in 12 to 15 hours. Intraperitoneal injection of 0.5 or 0.57 gram per kilo (dissolved in 0.5 per cent NaOH and 0.5 per cent Na_2CO_3 respectively) produced symptoms for about ten hours like those following the subcutaneous injections of similar amounts, and then the dogs promptly recovered. The alkali of the injected solutions did not cause the symptoms induced. The strengths of the solutions injected intraperitoneally ranged from 2.5 per cent to 8 per cent. In most cases it was about 4 per cent.

Intravenous injection of 0.7 or 0.85 gram per kilo (dissolved in 0.5 per cent Na_2CO_3) produced toxic symptoms and death after 8 and 4 hours respectively. Some of the toxic symptoms were vomiting, marked depression, restlessness, muscular twitching, extreme weakness, labored respiration, rapid and irregular heart beat and increased temperature. Intravenous injection of 0.4, 0.48 or 0.5 gram per kilo (dissolved in 0.5 per cent Na_2CO_3) produced toxic symptoms, like those just mentioned, for about 10 hours and then the dogs recovered. In the *fatal* cases (following the intravenous injection of mucoid solutions), no mucoid was excreted into the urine. In the animals that recovered from the

intravenous injections, the excretion of the mucoid was rapid. The strength of the solutions injected intravenously varied from 2 to 3.5 per cent. The alkali in the injected mucoid solutions did not cause the toxic effects observed.

The intravenous injection of mucoid solutions often produced a transient albuminuria. Congestion of the gastro-enteric tract was found in all dogs autopsied after intravenous injection.

Immunity to the toxicity of mucoid solutions was observed in two dogs. The serum of these animals gave marked "precipitin" tests.

A microscopic study of the tissues of animals poisoned with mucoid will be made. It is hoped that a "specific" stain for mucoid can be discovered.

Mucoid was detected in the blood after subcutaneous and intravenous injections.

A REAGENT FOR THE BIURET TEST.

By WILLIAM J. GIES.

From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

The author's reagent for the biuret test consists simply of 1 per cent KOH solution treated with sufficient 3 per cent CuSO solution (added little by little, with thorough shaking after each addition) to impart a slight though distinct blue color to the clear liquid. All the required copper is held in solution. The reagent has been in general laboratory employ for nearly a year and has given perfect satisfaction. Its use economizes time and material without loss of distinctness or definiteness of result, and offers numerous other advantages that will be detailed later.

On the nuclein metabolism of the rat. Alice Rohdé and Walter Jones. This *Journal*, vii, 237. 1910.

THE IODINE COMPLEX IN SPONGES (3,5-DIIODTYROSINE).

By HENRY L. WHEELER and LAFAYETTE B. MENDEL.

(From the Sheffield Chemical Laboratory and the Laboratory of Physiological Chemistry, Yale University.)

(Received for publication, November 3, 1909.)

The occurrence of iodine in living organisms has been known almost since the discovery of this element.[1] Little attention was devoted to its possible physiological rôle until after the discovery of iodine as a normal constituent of the thyroid gland by Baumann in 1895. This classic investigation, followed by Drechsel's isolation of "Jodgorgosäure" from the horny axial skeleton of a Gorgonian coral, awakened a new interest in the study of the organic iodine compounds. Henze[2] subsequently made a study of the substance described by Drechsel; he showed that it gives a xanthoprotein reaction and therefore is not iodaminobutyric acid as had been assumed by its discoverer.[3] The identification of the iodgorgoic acid was completed by Wheeler and Jamieson,[4] who established the fact that it is identical with 3,5-diiodtyrosine synthetically prepared by them. Aside from this no halogen-containing organic compound of known structure has yet been isolated from animal tissues.

Numerous circumstances exist to encourage the study of the organic halogen derivatives associated with animal tissues. The physiological action of thyroid tissue has been related to its iodine-containing complex, numerous investigations bearing witness to the comparative inefficacy of non-iodine-containing residues.

[1] Cf. v. Fürth: *Vergleichende physiologische Chemie der niederen Tiere*, p. 445; Aron: *Oppenheimer's Handbuch der Biochemie*, i, 67.

[2] Henze: *Zeitschrift für physiologische Chemie*, xxxviii, 60, 1903.

[3] Cf. Drechsel: *Zeitschrift für Biologie*, xxxiii, 90, 1896.

[4] Wheeler and Jamieson: *American Chemical Journal*, xxxiii, 365, 1905; see also Henze: *Zeitschrift für physiologische Chemie*, li, 64, 1907.

One recalls the observations of Reid Hunt[1] showing a close parallelism between the amount of iodine and the degree of physiological activity of preparations of the thyroid glands. There is evidence, furthermore, to show that iodine may be stored in organic form in animal organisms, and especially in the thyroids. From the biological and pharmacological point of view it is important to learn how the element administered in the form of inorganic iodides as a drug or otherwise, becomes synthesized into organic complexes.

In continuing the investigation of some of the problems here involved we have begun an examination of the organic substance of the sponges. The choice of this crude material was dictated by the experience of previous investigators who have pointed out the comparative richness of the albuminoid framework of these lower forms in halogens. As early as 1848 Vogel[2] suggested that the iodine found in sponges examined by him was present in an organic (not inorganic) form.

The iodine content of different types of sponges has been variously reported. The figures for the crude products in any case have no exact value because of the inherent impurities in the materials examined. Hundeshagen[3] selected for his interesting investigation a number of tropical and subtropical horny sponges, (*Luffaria*, *Aplysina*, *Verongia*), containing as high as 14 per cent of iodine in organic combination. He also showed the occurrence of other halogens (Cl, Br) in sponges and maintained that the assimilation of iodine in these organisms is a definite function, not an accident. As in the case of the corals[4] the rich occurrence of iodine is apparently associated with certain varieties. The bath sponges yield 1 to 1.5 per cent of iodine.

Hundeshagen's careful observations on the horn-like dried

[1] Hunt: *Journal of the American Medical Association*, xlix, 1323, 1907; Hunt and Seidell; *Bulletin* 47, Hygienic Laboratory, Public Health and Marine Hospital Service, 1909. The older literature is here reviewed in detail.

[2] Vogel: *Gelehrte Anzeigen der k. Bayerischen Akademie der Wissenschaften*, xxvii, 223, 1848.

[3] Hundeshagen: *Zeitschrift für angewandte Chemie*, 473, 1895.

[4] Cf. Mendel: *American Journal of Physiology*, iv, 243, 1900; Cook: *ibid.*, xii, 95, 1904; Mörner: *Zeitschrift für physiologische Chemie*, li, 32, 1907.

sponge skeletons investigated by him are of unusual significance. He noted that neither hot water nor organic solvents extract any notable amount of iodine. Superheated water or concentrated mineral acids liberate the iodine. Dilute mineral acids act in this way only gradually. Treatment with barium hydroxide dissolves the organic complex without liberating the halogen. The organically combined halogen is not precipitated by silver nitrate in nitric acid solution except on heating or addition of fuming nitric acid, whereupon silver iodide separates. An organic halogen compound can, however, be precipitated as a salt with silver, copper, or lead, if the alkaline solution of the sponge product is neutralized and the corresponding neutral salt of the metal added. Putrefaction appears to liberate iodine.

The properties here described in some detail suggest a close resemblance to the behavior of the diiodtyrosine[1] described by Wheeler and Jamieson and by Henze. Hundeshagen managed to obtain decomposition fractions successively richer in iodine, without attaining anything of uniform composition. He assumed, with unique chemical insight, that: "es müssten jodierte Amidosäuren vorliegen und zwar Jodamidofettsäuren oder Jodtyrosine oder beiderlei zugleich."

Harnack[2] examined ordinary bath sponges, containing 1.1 to 1.2 per cent of iodine. He demonstrated that in these, too, the halogen must exist in organic combination. In attempting to isolate this organic complex he learned that superheated steam destroys it completely so as to liberate iodine. He succeeded in obtaining a more concentrated product, albuminoid in character, which contained over 8.5 per cent of iodine and 9.4 per cent of nitrogen, and which he named "Jodspongin." Comparabl attempts had been made by Baumann.[3] Rosenfeld[4] prepared ae product having properties like Harnack's Jodspongin by boiling sponges with 12 per cent hydrochloric acid. The iodine content of the insoluble organic residue was 4.8 per cent.

[1] Wheeler and Jamieson: *loc. cit.*; Henze: *loc. cit.*

[2] Harnack: *Zeitschrift für physiologische Chemie*, xxiv, 412, 1898.

[3] Baumann: *Münchener medizinische Wochenschrift*, No. 14, 309, 1896; cf. Harnack: *loc cit.*, 419.

[4] Rosenfeld: *Archiv für experimentelle Pathologie und Pharmakologie*, xlv, 51, 1900.

An attempt to determine the nature of the iodine-yielding complex of the sponges has also been made by Scott.[1] This investigator claimed that hydrolysis with mineral acids as well as barium hydroxide liberates the iodine. By an undescribed treatment with sulphuric acid Scott transformed the spongin into a soluble product so that it could be subjected to pancreatic digestion, resulting finally in the complete disappearance of compounds yielding the biuret reaction. After removal of diamino acids with phosphotungstic acid and a leucine fraction by crystallization, an amorphous organic substance rich in iodine was obtained. The author states that it was separated from accompanying monamino acids "durch fraktionierte Krystallisation der Kupfersalze" and finally concentrated into a fraction soluble in alcohol. His "copper salt" could not have been that of iodgorgoic acid, as the latter is insoluble in water and alcohol and is non-crystalline; and since iodgorgoic acid is precipitated by phosphotungstic acid, as will be noted in the experimental part of this paper, it might be assumed that Scott was dealing with another iodine complex. It is more likely in our opinion, however, that the process of cleavage used by him did not yield amino acids and that Scott perhaps obtained some polypeptide combination, i. e., an incompletely hydrolyzed complex.

Halogen protein compounds have been prepared by several investigators.[2] They give the biuret reaction, but fail to respond to Millon's or the Hopkins-Cole test. It is probable therefore that the tyrosine (and perhaps tryptophane) groups are attacked by the halogen. It is significant in this connection that several of the earlier investigators of the organic basis of the sponges—the so-called spongin—failed to find tyrosine among the products of the hydrolytic cleavage of the albuminoid.[3] Even with the refined technique of the Fischer ester method, Abderhalden and Strauss[4] were unable to isolate tyrosine from spongin. This

[1] Scott: *Biochemische Zeitschrift*, i, 367, 1906.

[2] The literature on this topic has lately been compiled by Schryver: *The General Character of the Proteins*, p. 61ff., 1909.

[3] Cf. Städeler: *Annalen der Chemie*, cxi, 16, 1859; Zalocostas: *Comptes rendus de l'académie des sciences*, cvii, 252, 1888, reported finding a trace of tyrosine.

[4] Abderhalden and Strauss: *Zeitschrift für physiologie Chemie*, xlviii, 53, 1906.

corresponds with the statement of other investigators that spongin fails to give Millon's test, or at best affords a faint reaction.[1]

Strauss[2] has attempted to ascertain whether the failure to obtain Millon's reaction is due to a halogen substitution in the effective aromatic group. He subjected spongin to the action of superheated water with the object of liberating iodine. The tyrosine reaction was still negative with the products of the decomposition. Strauss therefore concluded that the halogen union was effected with other groups (phenylalanine?). The formation of iodoform during hydrolysis suggested to him the further possibility that the iodine might also be substituted in aliphatic complexes. Treatment with superheated steam would, however, be expected to decompose iodgorgoic acid without giving tyrosine.

Preliminary trials made by the writers with the common bath sponge demonstrated that boiling with barium hydroxide dissolves the so-called spongin without liberating any noteworthy amount of iodine from the organic complexes. We therefore adopted this method for the decomposition of the sponge albuminoids. Solutions so prepared gave no reaction with Millon's test. When treated with a solution of silver nitrate and nitric acid the clear fluid on warming suddenly becomes turbid just as the boiling point is reached; and the yellow solid causing the turbidity soon after separates as a heavy precipitate of silver iodide. This reaction is exceedingly delicate for diiodtyrosine; *p*-iodphenylalanine, on the other hand, can be boiled in the same way without separating silver iodide.[3] This behavor of the solutions of the sponge products, together with the typical resistance of the iodine-compound toward treatment with barium hydroxide pointed strongly to the existence of a complex similar to, if not identical with, the iodgorgoic acid of the corals. It was found possible to separate a silver compound rich in iodine

[1] Cf. Krukenberg: *Zeitschrift für Biologie*, xxii, 241, 1886; Harnack *Zeitschrift fur physiologische Chemie*, xxiv, 417, 1898, pronounced "unsicher" the Millon's test with the "Jodspongin" prepared by himself.

[2] Strauss: *Studien über die Albuminoide mit besonderer Berücksichtigung des Spongins und der Keratine*, Heidelberg, 1904; quoted from *Maly's Jahresbericht für Tierchemie*, xxxiv, 34, 1904.

[3] Wheeler and Clapp: *American Chemical Jounal*, xl, 459–60, 1908.

and soluble in excess of nitric acid, such as was originally obtained by Drechsel and later by Henze. Our earliest attempts to isolate a crystalline product from this silver salt failed. The silver-free residues assumed a varnish-like character on concentration and did not crystallize. They were still resistant to decomposition by barium hydroxide at this stage.[1] By fractionation of the product with phosphotungstic acid we have now succeeded in obtaining 3,5-diiodtyrosine in abundance in typical crystalline form. The phosphotungstic acid precipitate contains other amino acids besides diiodtyrosine, which prevent the crystallization of this substance after a certain amount has separated. The thick mother liquor responds to the silver nitrate-nitric acid test and gives an abundance of silver iodide—apparently enough to account for all of the iodine present.

The ease with which iodine enters into combination with the aromatic group suggests a number of considerations respecting the function of the latter in proteins as they exist in the diverse tissues. Thus in the thyroid the halogen is found in the globulin alone, not in the nucleoprotein of the gland. The occasional accumulation of iodine in keratin-yielding tissues (skin, hair) which are as a rule comparatively rich in the tyrosine complex is suggestive in any event.[2] That it is the latter group with which the halogens ordinarily react seems most probable from a purely chemical standpoint. Tyrosine readily combines with iodine while phenylalanine does not,[3] under conditions which might be approximated in the animal organism. The behavior of tryptophane under similar circumstances remains to be investigated. Neuberg[4] obtained an *amorphous* iodine derivative of tryptophane by treatment of the latter with iodine in alkaline solution.

[1] One of us earlier had a similar experience in attempting to isolate iodgorgoic acid from Gorgonian corals. (Cf. Mendel: *American Journal of Physiology*, iv, 243, 1900.) This behavior has doubtless led others also to assume that the iodine is present in some form different from that originally isolated by Drechsel.

[2] Cf. Justus: *Archiv für pathologische Anatomie*, clxxvi, 1, 1904; Howald: *Zeitschrift für physiologische Chemie*, xxiii, 209 (hair), 1897.

[3] Oswald: *Archiv für experimentelle Pathologie und Pharmakologie*, lx, 126, 1908.

[4] Neuberg: *Biochemische Zeitschrift*, vi, 276, 1907.

The fact that 3,5-diiodtyrosine is contained in sponges requires that Harnack's formula for iodospongin, $C_{56}H_{87}IN_{10}S_2O_{20}$, must at least be doubled. It is curious that 3,5-diiodtyrosine is precipitated by phosphotungstic acid, while tyrosine is not. In this connection it may be stated that we have examined 3,5-dibromtyrosine and find that this acid is also precipitated by phosphotungstic acid. In fact, the precipitants, as far as examined, that precipitate the iodine acid also precipitate the bromine compound. The acids show a close similarity in properties, as might be expected. When dibromtyrosine is warmed with silver nitrate and nitric acid, silver bromide separates.

EXPERIMENTAL PART.

The material was composed of specimens of ordinary bath sponges, from the Florida coast. They were soaked in water, washed and again dried. An analysis showed 0.69 per cent of iodine in this crude product. After the preliminary trials already mentioned the sponges were hydrolyzed as follows: 400 grams were heated to boiling with 3 liters of barium hydroxide solution containing about 800 grams of the hydrate; the heating was continued 8 hours, the volume being kept constant. No free iodide was present at this stage; but the fluid showed an abundance of organically combined halogen which precipitated as silver iodide when the solution was heated with nitric acid and silver nitrate (after removing the silver sulphide formed.) The heating was continued about 40 hours longer. On cooling the solution was mixed with toluene and then stood for about three months before receiving further attention. Considerable sediment separated. This consisted mostly of barium hydroxide and the barium salts of aspartic acid; it contained some iodine and was treated separately in the same manner as the solution. The latter was precipitated with silver nitrate until an excess of silver was present and the filtrate, on boiling with nitric acid, no longer gave a turbidity. The silver salts, still moist, were treated at ordinary temperature with dilute nitric acid, at first 1 part of concentrated nitric acid with 4 parts of water, and finally with concentrated nitric acid diluted with an equal volume of water. The acid solution was filtered from silver sulphide and

then carefully neutralized with ammonia. The precipitate, but not the filtrate, gave a residue of silver iodide when boiled with nitric acid. The precipitate was washed thoroughly with water, then suspended in water and decomposed by hydrogen sulphide. On evaporating the solution on the steam-bath a brown tar was obtained. This was taken up in water and allowed to evaporate spontaneously. The thick syrup deposited crystals which filtered very slowly on the pump. When dried on a porous plate the material was light gray and weighed 12.0 grams. It was boiled with water and animal charcoal and then recrystallized from water. It formed colorless spikes or hard, brittle, prismatic crystals which were free from iodine and sulphur. A nitrogen determination agreed with the calculated content for *aspartic acid*:

	Calculated:	Found:
N	10.52 per cent.	10.53 per cent.

The mother liquor from the above was allowed to stand for some time, but no crystals separated. It was then decided to precipitate the solution with phosphotungstic acid. For this purpose 10 cc. of concentrated sulphuric acid were diluted with 190 cc. of water and 80 cc. of this acid were taken to dissolve the thick syrup. Then 100 grams of phosphotungstic acid in 70 cc. of the acid were added to the solution of amino acids. This produced a doughy precipitate which it was impossible to wash in a satisfactory manner. The whole was allowed to stand over night. The precipitate, still doughy, was washed superficially with 50 cc. of the above acid and then heated on the steam-bath with a solution of barium hydroxide in excess. The operation being repeated, the solution was precipitated with carbon dioxide and the precipitate thoroughly washed. The filtrate on evaporation gave a very soluble varnish. *This proved to contain the barium salt of the iodine compound.* It was dissolved in a little water and dilute sulphuric acid was carefully added until no further separation of barium sulphate took place, or at least until only a small amount of barium remained in the solution. The light yellow solution was placed in a desiccator over sulphuric acid and allowed to stand over night. The next morning a sandy crystalline mass had separated which was seen under the micro-

scope to consist of thin, yellowish white plates. This first crop of crystals weighed exactly one gram. When a portion was warmed with nitric acid and silver nitrate, silver iodide separated in abundance. From the mother liquor 1.2 grams more of this crystalline material were obtained; then a gum separated enclosing the crystals so that they could not be filtered. The first crop of crystals was recrystallized from water and Prof. George S. Jamieson very kindly made an iodine determination using the method previously described.[1] 0.1260 gram of substance gave 0.1360 gram AgI.

	Calculated for diodtyrosine, $C_9H_9O_3NI_2$:	Found:
I	58.66 per cent.	58.32 per cent.

The material, when rapidly heated, melted at 213° with strong effervescence, turning brown-black. Abderhalden and Guggenheim[2] give the melting point of 3,5-diiodtyrosine at 213°. A sample of synthetic 3,5-diiodtyrosine prepared in this laboratory by Dr. S. H. Clapp melted at 213° and when a portion of our material was mixed with it the melting point was not altered. The compounds are therefore identical.

The material when warmed with Millon's reagent gave no red color. A portion was dissolved in strong hydriodic acid and evaporated to dryness on the steam-bath. After this reduction the aqueous solution gave a strong test for tyrosine with the above reagent. The appearance, properties, solubility, etc., of the above crystals agreed in every respect with those of 3,5-diiodtyrosine.

We therefore conclude that the above results definitely prove that the iodine complex in sponges is 3,5-diiodtyrosine, or the so-called iodgorgoic acid.

[1] Wheeler and Jamieson: *loc. cit.*

[2] Abderhalden and Guggenheim: *Berichte der deutschen chemischen Gesellschaft*, xli, 1237, 1908.

ON THE PREPARATION AND PROPERTIES OF IODO-MUCOIDS.[1]

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(Received for publication, November 1, 1909.)

Introduction.

In continuation of the work done in this laboratory on the mucoids, by Dr. Gies and collaborators, I have lately prepared and analyzed products formed by the action of iodin on tendomucoids. These iodo-products, to which the name iodo-mucoid may be given, contain about 14 per cent of iodin. Similar derivatives of other proteins have been prepared and described by several investigators, the iodin contents of which varied from about 8 to 22 per cent.[2]

It has been assumed by both Blum[3] and Hofmeister[4] that the production of a particular iodo-protein depends upon the substitution of iodin for *hydroxyl* radicals in one or more of the numerous aromatic nuclei in each of the molecules of the corresponding protein. These observers came to this conclusion because hydriodic acid is invariably produced in the process of iodo-protein preparation. More recently the studies of Henze[5] led to the conclusion that the iodin in such a compound is substituted for hydroxyl in the *tyrosin* nucleus of the molecule. This probability was also suggested by Hofmeister on the ground that the iodized protein no longer responded positively to the Millon test. After the introduction of iodin into tendomucoids these proteins,

[1] An abstract of a preliminary report appeared in *Science*, xxv, p. 457, 1907.

[2] Lepinois: *Journal de Pharmacie et de Chimie* (6), v, p. 561, 1897.

[3] Blum: *Journal für praktische Chemie* (2), lvi, p. 393, 1897.

[4] Hofmeister: *Zeitschrift für physiologische Chemie*, xxiv, p. 167, 1896.

[5] Henze: *Zeitschrift für physiologische Chemie*, li, p. 66, 1907.

also, no longer respond to either the Millon or the Adamkiewicz test. It is probable that, besides being substituted for hydroxyl, iodin combines with protein products in other ways.

Preparation of Tendomucoids.

The tendomucoid products for this investigation were extracted from Achilles tendons of oxen with half-saturated lime water.[1] The tendon segments were cut into thin cross sections, which were then well washed with running water. The lime water extracts, after filtering, were slightly acidified with 0.2 per cent hydrochloric acid and the precipitated mucoids washed several times by decantation with water and finally collected on hardened filter papers. A large portion of this mucoid material was used for the preparation of iodo-mucoids, as described below; the rest was reserved for comparative analysis. The latter portion was thoroughly washed successively with 50 per cent alcohol, 95 per cent alcohol and finally with ether, and then dried at 110° C. to constant weight. Elementary analysis gave the following percentage results, the average of two closely agreeing figures in each instance (oxygen by difference):

C	H	N	S	O
48.02	6.63	12.50	2.18	30.67

Preparation of Iodo-mucoids.

After several trials the following procedure for the preparation of iodo-mucoids was found to yield the largest amount of precipitable material; 50 grams of mixed tendomucoids,[2] obtained by the process described above, were dissolved in sufficient 0.5 per cent sodium carbonate solution to make a complete and contentrated though somewhat opalescent solution. Into this solucion was dropped from time to time and in small amounts, as often as the previously added portion vanished, a total of 16 grams of finely pulverized iodin. Frequent stirring of the mixture favored the reaction. The mixture was kept at 50° C. for

[1] Cutter and Gies: *American Journal of Physiology*, vi, p. 155, 1901; also Gies and collaborators: *Biochemical Researches*, i, p. 163 (Reprint No. 5), 1903.

[2] Calculated for dry material.

several days, with repeated stirrings and additions of dilute sodium carbonate to neutralize the acidity that resulted from the formation of hydriodic acid. The clear liquid was then filtered and the iodo-mucoid products thrown down by the addition of dilute hydrochloric acid. The iodo-mucoids were thus precipitated in large, heavy, red flocks, much resembling freshly precipitated ferric hydroxid in general appearance. The precipitate was allowed to settle, then washed several times with water by decantation and finally isolated by filtration. The precipitate was further thoroughly treated successively with 50 per cent alcohol, 95 per cent alcohol, and ether. The treatment with 95 per cent alcohol was continued till the washings showed no further evidence of the presence of free iodin in the precipitate. The product was then dried at 110° C. The yield was 21.5 grams.

Dry mixed iodo-mucoid, as thus obtained, is a light, yellowish powder. The product is odorless and tasteless. It readily dissolves in dilute sodium carbonate or other alkaline liquids to form solutions that in general are as frothy as those of tendomucoid itself. Upon acidifying such an alkaline solution with dilute nitric acid, the iodo-mucoids are reprecipitated and the clear filtrate will not yield a precipitate wilth silver nitrate. The iodin content of this material cannot be due to any adherent iodid.

Analysis has shown that iodo-mucoids may be reprecipitated practically unaltered. Iodo-mucoids do not respond to either the Adamkiewicz test or the Millon test. The results of the xanthoproteic and biuret tests are positive, as is also that of the Molisch test. After hydration of iodo-mucoids with dilute acid, the solution readily reduces Fehling solution. Iodin is easily detected after fusion of iodo-mucoids with sodium, or by the copper-oxid flame test.

Analysis of Iodo-mucoids.

For the quantitative determination of *iodin* in the products the following method was employed. The substance, previously dried to constant weight at 110° C., was weighed into a platinum crucible and moistened with a few drops of a 10 per cent potassium hydroxid solution. The mass was then covered with 40 parts by weight of a mixture consisting of 1 part of sodium car-

bonate and two parts of potassium nitrate, and subjected to complete fusion and oxidation. The cooled mass was dissolved in warm, very dilute nitric acid, and then boiled for a minute to expel nitrous acid. After cooling, the iodin was precipitated with $\frac{N}{50}$ silver nitrate solution and weighed as silver iodid. The amount of iodin was also approximately determined volumetrically as a check. *Carbon* and *hydrogen* were determined by combustion, *sulfur* by the caustic alkali fusion process. Analytic details are appended:

1, *a*....0.3294 gram of substance gave	0.0857 gram of	AgI	
b....0.3015 " " "	0.0787 "	AgI	
2, *a*....0.1225 " " "	0.0775 "	H_2O; and 0.1965 gram of CO_2	
b....0.1454 " " "	0.0915 "	H_2O; and 0.2327 gram of CO_2	
3, *a*....0.3902 " " "	0.0480 "	$BaSO_4$	
b....0.4663 " " "	0.0553 "	$BaSO_4$	

Nitrogen was determined by the Kjeldahl method. Samples weighing 0.1335 gram and 0.1912 gram were taken for two determinations of nitrogen content. *Oxygen* was calculated by difference. The percentage data are appended:

SAMPLE.	C	H	N	S	I	O
1..........................	43.75	7.07	12.56	1.47	14.06	21.09
2..........................	43.66	7.04	12.40	1.67	14.10	21.18
Average....................	43.71	7.05	12.48	1.57	14.08	21.14

In order to determine whether the iodo-mucoids suffered loss of iodin by reprecipitation, a few grams of the product referred to above were dissolved in 0.5 per cent sodium carbonate solution, reprecipitated with a 0.2 per cent solution of hydrochloric acid and further purified by the process already described. Analysis disclosed the following iodin content:

Substance taken....0.1200 gram = 0.0315 AgI = 14.18 per cent. I.
Substance taken....0.1268 gram = 0.0334 AgI = 14.24 " I.

The change in the iodin figures is too slight to warrant the conclusion that much of an alteration occurred.

Iodization of the Mycoids in Successive Extracts of Tendon.

In the several preparations of mixed iodo-mucoids, which were made by the action of iodin upon alkaline solutions of mixed tendomucoids, it was noticed that the total yield of iodo-mucoids in each case was not as great as might have been expected. It was also observed on evaporating to dryness the neutralized liquid from which the iodo-mucoid had been precipitated with acid, that a residue of an organic nature was obtained which was quite soluble in water and precipitable by alcohol. The nature of these residues has not been determined. Similar behavior on the part of tendomucoids themselves had already been noticed.[1]

It was thought that mucoids obtained from tendons by *fractional extraction* might give rise to products with different contents of iodin. Accordingly, further inquiry along these lines was made as follows: A lot of Achilles tendons from oxen was prepared for extraction with lime water by the process already described. Four successive extracts were made with half-saturated lime water, each extraction lasting 24 hours.[2] In this way four samples of tendomucoid material were obtained, which were separately redissolved and reprecipitated once prior to further treatment with iodin. Each purified tendomucoid product was promptly dissolved in 0.5 per cent sodium carbonate solution, treated with an excess of finely powdered iodin and kept at 37° C. for 24 hours. The clear solutions were then filtered and the iodo-mucoids precipitated from the filtrates with dilute (2 per cent) acetic acid. The acid filtrates were saved for the further treatment to be described. The iodo-mucoid precipitates from each tendomucoid product were given identical treatment with alcohol and ether, and finally were dried at 110° C. for analysis. The following percentage analytic data were obtained:

Iodized Fraction.	Iodin per cent.	Nitrogen per cent.
1	14.20	14.09
2	14.26	14.03
3	14.18	13.27
4	14.00	13.46

[1] Gies: *Science*, xxv, p. 463, 1907.
[2] Cutter and Gies: *loc. cit.*

These variations are hardly decided enough to warrant the positive conclusion that more than one mixture of iodo-mucoids was under examination, yet they do point to the probability, in line with the deduction by Cutter and Gies, that tendon contains more than one mucoid.

The acid filtrates from the four iodo-mucoid preparations that are referred to above were neutralized with sodium carbonate, evaporated on a water bath to a small volume and poured into an excess of alcohol. The precipitates were washed with 95 per cent alcohol and ether to free them from water and iodin. The products closely resembled the previously prepared and corresponding iodo-mucoids. They possessed protein properties, though they did not respond positively to the Millon or Adamkiewicz tests. They contained iodin and, after hydration with acid, reduced Fehling solution.

These particular results suggest that in the preparation of the iodo-mucoid products, fragments of the tendomucoid molecules were split off, which in many respects resembled the original mucoids, but differed from them especially in their solubility. Whether such a cleavage is brought about by the action of iodin alone, or merely as a result of acidifying the iodized product, cannot be stated at present. Of course it is quite possible that acid reprecipitation of iodo-mucoid mixtures, such as those at hand in these experiments, may result merely in *mechanical* separation of the products under consideration.

Further studies of iodo-mucoids in this and other relations are in progress in this laboratory under Dr. Gies' direction.

SUMMARY.

1. Iodo-mucoid products were prepared by the action of iodin on tendomucoids in dilute sodium carbonate solutions. The products were modified proteins possessing most of the properties of mucoids.

2. Iodo-mucoids in the dry state are yellow, odorless and tasteless powders containing approximately 14 per cent of iodin in organic combination. Iodo-mucoids do not appear to be decomposed by acid reprecipitation from dilute alkaline solutions, although this matter has not been settled.

In conclusion I wish to express my appreciation of the suggestions and encouragement I have received from Dr. William J. Gies.

LACTIC ACID IN THE AUTOLYZED DOG'S LIVER.

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(Received for publication, November 1, 1909.)

In a previous communication[1] the discovery of a peculiar anaërobic bacterium in twenty-two out of twenty-four healthy dogs' livers was reported. This organism, which does not grow on usual culture media, was found to exercise both a putrefactive and fermentative action on the liver tissue as well as liver extract, resulting in the production of a large amount of carbon dioxide and a strong acid reaction. At that time the writer raised a question[2] as to the primary origin of lactic acid in autolyzed liver tissue in these words:

"The occurrence of fermentation lactic acid[3] in the dog's liver after standing a while, which has been observed by previous investigators, was questioned by Saiki, as being a product of autolysis since it may be possible that this peculiar liver bacterium is responsible for this kind of lactic acid. Portions of the livers from Dog IV and Dog XII which showed no bacteria were analyzed for lactic acid, after 48 hours incubation at 37° C. The zinc salt in prismatic and needle form was obtained only in a small quantity by using the usual laboratory method, and the amount was not sufficient to determine the character of the salt isolated. Both the difficulty of getting bacteria-free dog's liver and the very small amount of lactic acid produced in such a liver, were the obstacles which prevent the solution of the above question at the present time."

The living liver cell is alkaline to litmus and acid to phenolphthalein. This reaction becomes distinctly acid very soon after

[1] Wolbach and Saiki: *Journal of Medical Research*, xxi, p. 267, 1909.

[2] Cf. footnote in article by Jackson: *Ibid.*, xxi, p. 283, 1909.

[3] Cf. Ekunina: *Journal für praktische Chemie*, xxi, p. 478, 1880; Morishima: *Archiv für experimentelle Pathologie und Pharmakologie*, xliii, p. 217, 1899; and also Magnus-Levy: Hofmeister's *Beiträge*, ii, p. 261, 1902.

death and the presence of lactic acid has frequently been demonstrated. Before it came to be recognized as a well established fact that lactic acid is a normal constituent of liver, the possibility of a *post mortem* origin had been suggested.[1]

It is, however, generally regarded that the lactic acid[2] in vertebrate muscle, brain, lymph glands, suprarenal glands, kidney, thymus, spleen, pancreas, gastro-intestinal wall and normal liver as well as blood, vitreous humor, cerebro-spinal fluid and also in the urine, etc., is sarco-lactic acid. Fermentation lactic acid has been found *intra vitam* in experimental arsenic poisoning, viz: in the dog's blood by H. Meyer[3] and in rabbit's urine by Araki.[4] It has been proved however by subsequent investigators[5] that lactic acid in Meyer's case is not of the fermentation but of the sarco-lactic variety. The presence of fermentation lactic acid in the muscle[6] is very uncertain and only minimum in quantity if any.

Since the footnote[7] was written, another dog's liver (Dog XXV) has been obtained, which showed a perfectly sterile condition after being allowed to stand forty-eight hours in an incubator at 38° C. This specimen was analyzed for lactic acid and the zinc salt was prepared according to the customary method.

The zinc salts obtained, including those from previous dogs' livers (Dog IV and Dog XII) are as follows:

	WEIGHT OF LIVER USED.	ANHYDROUS ZINC SALT OBTAINED.	LACTIC ACID IN 100 GRS. OF FRESH LIVER CALCULATED.
	grams.	*grams.*	*grams.*
Dog IV .... about	20	trace	
Dog XII	45	0.431	0.701
Dog XXV	110	0.526	0.350

[1] Cf. Hoppe-Seyler: *Physiologische Chemie*, p. 718, 1881.

[2] For the literature on this subject, see review by Moriya; *Zeitschrift fur physiologische Chemie*, xliii, p. 397, 1905; and Mandel and Lusk, *American Journal of Physiology*, xvi, p. 129, 1906.

[3] H. Meyer: *Archiv für experimentelle Pathologie und Pharmakologie*, xvii, p. 304, 1883.

[4] Araki: *Zeitschrift für physiologische Chemie*, xvii, p. 331, 1893.

[5] Morishima: *loc. cit.*

[6] Cf. Heintz: *Annalen der Chemie und Pharmacie*, clvii, p. 314, 1871; Siegfried; *Berichte der deutsch. chem. Gesellschaft*, xxii, p. 2711, 1889.

[7] Jackson: *Journal of Medical Research*, *loc. cit.*

These were united, purified with animal charcoal and recrystallized. The recrystallized salt was then dissolved in water, evaporated to a small volume and a small amount of absolute alcohol added. Upon standing, crystallization took place. The first crystals were isolated from the mother liquid, and these analyzed separately from the second crop. The former proved to be fermentation lactic acid and the latter sarco-lactic acid.

I. Analysis of first fraction:

0.3186 gram of the zinc salt lost 0.0575 gram at 110° C.

2.61 per cent aqueous solution of the anhydrous salt in 100 mm. tube showed no polariscopic rotation.

0.2611 gram of the anhydrous salt gave 0.0868 gram ZnO. Therefore,

	Analysis per cent.	Calculated for $(C_3H_5O_3)_2Zn + 3H_2O$. per cent.
Water of crystallization	18.05	18.18
Zinc content of anhydrous salt	26.65	26.75

II. Crystals from second fraction.

0.3791 gram of the zinc salt lost 0.0500 gram at 110° C.

−0.24° was observed with 3.29 per cent aqueous solution of the anhydrous zinc salt in 100 mm. tube.

0.3291 gram of the water-free crystals gave 0.1099 gram of ZnO. Hence,

	Analysis.	Calculated for $(C_3H_5O_3)_2Zn + 2H_2O$.
Water of crystallization	13.18 per cent.	12.90 per cent.
Zinc content of anhydrous salt	26.79 "	26.75 "
$[\alpha]_D^{20}$	−7.29°	−6.5° to −8.7°

In the above described liver autolyses no actual determinations of the glycogen content were made and no foreign glycogen was added. To determine the sterile condition of the liver tissues it was found necessary to incubate this organ in large pieces without previous hashing or any other complicated operation. The animals however were well fed and each of them showed positive evidence of rich glycogen content, as a part of the liver tissue was used to prepare culture media.

It is evident that lactic acid as an autolytic product of normal dog's liver is largely sarco-lactic acid as in other organs. A

rather small amount of fermentation lactic acid is, likewise, observed in even the strictly sterile condition. From 175 grams of the liver tissue autolyzed 0.9671 gram of the anhydrous zinc salt was obtained in the total lactate determination; 0.2611 gram of this was found to be fermentation lactic acid.

LIQUID EXTRACTION WITH THE AID OF SOXHLET'S APPARATUS.

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(Received for publication, November, 1, 1909.)

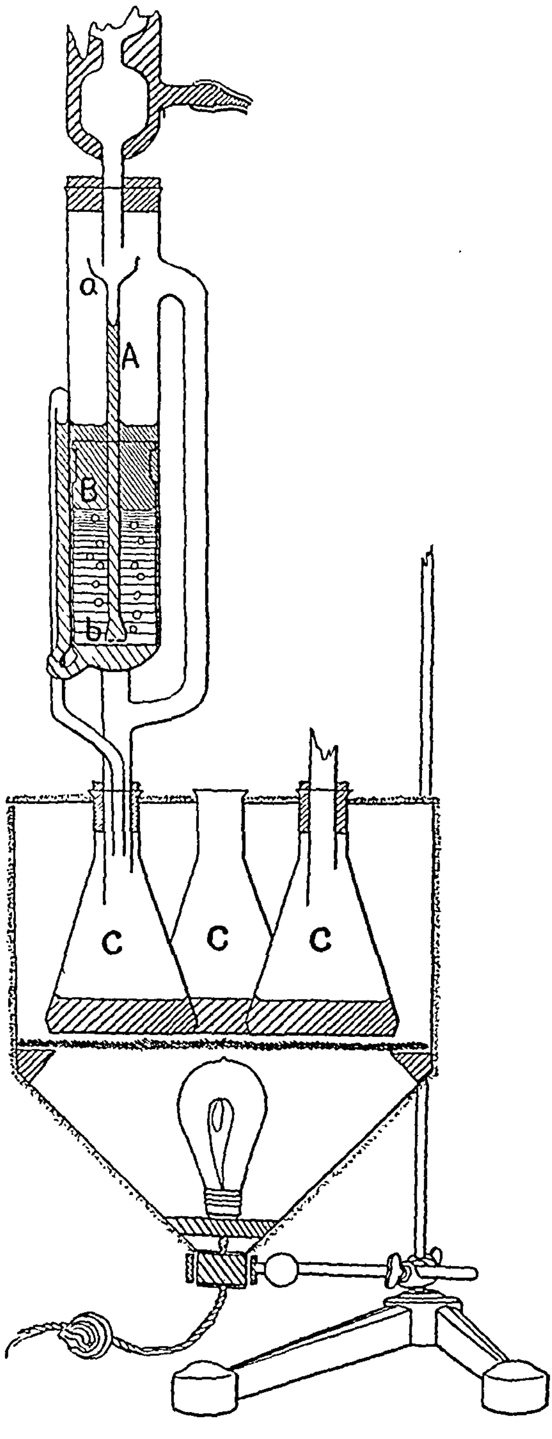
To extract ether-soluble constituents from liquid material without using the shaking method, a specific apparatus is required. Those described by Schwartz, by Kumagawa-Suto, and by Steudel are most widely employed. Soxhlet's apparatus accompanied by a glass tube and a small bottle, however, can be utilized for such extraction.

The method is as follows:

Take a small glass tube (*A*) (3 mm. in diameter), form a funnel shape (*a*) to one end by blowing, and make two small holes (*b*) (1 mm. in diameter) at the other end by union of opposite points of the circumference of the glass tube. The bottle (*B*) should be of a diameter that can be readily inserted in Soxhlet's apparatus and about the length of the siphon.

The extracting material is first placed in the bottle which is then inserted with the aid of a pair of long forceps into Soxhlet's apparatus which has already been connected with the flask (*C*) containing the ether for extraction. The glass tube (*A*) is now placed in the bottle (*B*) and the entire apparatus connected to the condenser so that the upper end of the glass tube (*A*) becomes the receiver of the condensed ether.

The apparatus, so modified, proved to be very convenient for practical work, and very satisfactory results were obtained. It has been found that the quantities of lactic acid present in tissue extracts can be completely extracted in fifteen hours when this method is employed. Usually the lactic acid solution was concentrated to about twenty-five cubic centimeters before extraction. One thirty-two candle power incandescent light furnishes sufficient heat for three simultaneous extractions when the electric bulb and flasks containing the ether are placed within a tin box covered with asbestos and fitted with an asbestos lid.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A STUDY OF THE CHEMISTRY OF CANCER.

II. PURIN BASES, CREATIN AND CREATININ.

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(Received for publication, November 1, 1909.)

Since malignant growths consist of cells rich in protoplasm, it seems quite natural that the nitrogenous constituents first attracted investigators to a study of their chemical character. In an investigation of the various protein constituents Wolff[1] observed a high albumin content, as compared with globulin, in the press juice of cancerous tissue, though no qualitative differences of proteins were detected between cancer cells and normal cells. Utilizing our modern chemical knowledge of the protein decomposition products, Bergell and Dorpinghaus[2] determined the various amino-acids, with the idea of learning the nature of the proteins of cancer and sarcoma. The results of their analyses, however, disagreed somewhat with those of Neuberg,[3] who, with the same end in view, subsequently determined the amount of tyrosin, leucin, glutamic acid and glycocoll in tumor protein. The attempts to determine the character of the protein, whether simply transported into the cell or newly formed as a specific tumor protein, have been unsuccessful.

From the morphology of the large nuclei in cancer cells one would look for a high percentage of nucleoprotein and this was observed by Petry.[4] According to Bang[5] the nucleoprotein is

[1] Wolff: *Zeitschr. f. Krebsforschung*, iii, p. 95, 1905; *Medizinische Klink*, (1), p 13, 1905.
[2] Bergell and Dorpinghaus: *Deutsch. med. Woch.*, xxxi, p. 1426, 1905.
[3] Neuberg: *Arb. a. d. Path. Inst. zu Berlin*, p. 593, 1906.
[4] Petry: *Zeitschr. f. physiol. Chem.*, xxvii, p. 398, 1899.
[5] Bang: Hofmeister's *Beiträge*, iv, p. 368, 1903.

specific and characteristic of mother tissue. Furthermore, the presence of intracellular proteolytic enzymes in such cells seems very probable, as is indicated by the autolytic products which are formed in tumor tissue.

It was the author's intention in the present study of malignant growths to determine the purin bases, as these might demonstrate a resemblance to normal tissue of similar origin or at least express the specific protein splitting action according to the source of the tumor.[1]

The tumors were all surgical specimens which were analyzed shortly after removal. Owing to the necessity of getting sufficient material to determine the occurrence of the purin bases in tumors in general, several specimens were put together.

Experiment I.

Material consists of	Grams.
1. Carcinoma of ovary	61
2. Carcinoma of breast	69
3. Carcinoma of rectum	8.5
4. Carcinoma of rectum	9
5. Carcinoma of breast	3.8
6. Carcinoma of breast	50
	201.3

These were extracted several times with fresh boiling water, the coagulable proteins removed by heating with acetic acid and the purin bases precipitated by the Krüger-Schmid method. Only a trace of copper precipitate, however, was observed and it was found impossible to isolate individual purin bases and therefore only total nitrogen was determined after reprecipitation by the same method. Nitrogen found was 0.0019 gram in 201.3 grams or 0.001 per cent of fresh tissue.

This clearly emphasizes the fact that in fresh cancerous tissue purin metabolism apparently is not the principal factor in the marked disintegration which has often been suggested as of importance in explaining the systemic manifestations of malignant growths.

[1] This work has not been completed owing to the difficulty in obtaining the proper material for analysis and only a preliminary statement is made here as the writer is leaving this laboratory.

In the second series of specimens, however, the total purin bases and uric acid were determined, and uric acid, adenin, hypoxanthin and trace of guanin and xanthin were obtained.

Experiment II.

Specimens analyzed were

	Grams.
1. Carcinoma of breast	4
2. Carcinoma of ovary, uterus and rectum	43
3. Carcinoma of ovary	508
4. Carcinoma of breast	17
	572

Cancerous tissue contains a considerable amount of fat and lipoid substances. After removal of these substances by extraction[1] with boiling alcohol and then with a mixture of alcohol and ether, the specimens were boiled with 5 per cent sulphuric acid on the sand-bath until no biuret reaction was obtained. Then the Krüger-Schmid method for the determination of purin bases was applied. The precipitation was twice repeated. The results of analysis are as follows:

	Gram.	
Uric acid	0.1391	(or 0.024 per cent of tumor tissue).
Guanin	trace	(a few crystals as guanin hydrochloride).
Adenin	0.1866	(as adenin picrate).
Hypoxanthin	0.0190	(as hypoxanthin nitrate).
Xanthin	trace.	

These figures suggest the occurrence of guanase and xanthoxidase and the absence of adenase, quite in correspondence with more recent observations of Winternitz and Jones on human tissues.[2]

[1] These extracts were combined and freed from alcohol and ether. It was made alkaline in reaction to sodium carbonate and extracted with ether. It was then acidified with phosphoric acid and extracted with ether for lactic acid, which was isolated as the zinc salt. The anhydrous zinc salt obtained from 572 grams of tissue was 0.850 gram (or 0.108 per cent of fresh tumor tissue). The lactic acid present in the tumors was that of *d*-modification.

[2] Winternitz and Jones: *Zeitschr. f. physiol. Chem.*, lx, p. 180, 1909.

Creatin and creatinin were determined according to Folin's colorimetric method.[1] The results are as follows:

Creatinin (before boiling with acid) 0.016 per cent of fresh tissue.
Total creatinin (after boiling with sulphuric acid) 0.019 per cent of fresh tissue.

Postmortem change cannot be considered here as the specimens employed were analyzed very shortly after removal. It is a very interesting fact that creatinin was obtained but practically no creatin.

According to Mellanby[2] and van Hoogenhuyze and Verploegh[3] a very high creatin elimination is observed in carcinoma of liver. The former has also found that the creatinin output is diminished in the case of liver carcinoma while the latter obtained almost no creatin in the urine of individuals suffering from carcinoma of stomach. If the creatin and creatinin metabolism has any connection with liver, this can easily be explained.

[1] Cf. van Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, xlvi, p. 432, 1905.
[2] Mellanby, *Journ. of Physiol.*, xxxvi, p. 447, 1908.
[3] van Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, lviii, p. 161, 1908.

A NOTE ON THE ESTIMATION OF PURIN NITROGEN IN URINE.

By STANLEY R. BENEDICT and TADASU SAIKI.

(Received for publication, November 1, 1909.)

During a long series of determinations of purin nitrogen in urine by the Krüger-Schmid method,[1] we found many instances where *total* purin nitrogen by this method was lower than uric acid nitrogen alone by the Folin-Shaffer method.[2] An investigation showed that the error lay in the Krüger-Schmid method and not in the procedure for uric acid.

This tendency of the Krüger-Schmid method to give low and irregular results we have found to be possible of correction by carrying out the first precipitation in a decidedly acid solution, instead of in the nearly neutral reaction which obtains in the urine. The addition of 20 cc. of glacial acetic acid, or an equivalent quantity of dilute acetic acid, for each 300 cc. of urine employed, is advised. This addition of acid should be made as preliminary to the first precipitation.

After making this slight change in technique, consistently satisfactory results were obtained, the duplicate determinations showing a closeness of agreement which could not be secured without addition of the acid, and the figures obtained were always reasonably higher than those for uric acid nitrogen alone.

[1] Krüger and Schmid: Hoppe-Seyler u. Thierfelder, *Handbuch der physiologisch- und pathologisch-chemischen Analyse*, p. 435, 1903.

[2] Folin: *The American Journal of Physiology*, xiii, p. 49, 1905.

ON THE NEUTRALITY EQUILIBRIUM IN BLOOD AND PROTOPLASM.

By LAWRENCE J. HENDERSON.

(From the Laboratory of Biological Chemistry of the Harvard Medical School.)

(Received for publication, November 4, 1909.)

In a recent paper Brailsford Robertson[1] has discussed acid-base equilibrium in the animal body. His paper deals very largely with matters already treated by me,[2] and several divergences of opinion call for further consideration.

In the first place I gladly accept the correction of an error which Robertson has pointed out in one of my papers.[3] I have there calculated the approximate magnitude of the variation in the amount of sodium combined with proteins in the blood plasma. The corresponding change in reaction of the blood was between perfect neutrality and the ordinary alkalinity, and the result was erroneously regarded as the total sodium-protein content of the blood. Of course there is to be added to this, which represents the *variable* sodium-protein content of the blood, that moiety of sodium which is combined with the plasma proteins at neutrality. This latter quantity is at present undetermined.

In all other respects I am unable to agree with Robertson's arguments, and I wish to take up some of his points in order.

At p. 317 Robertson says: "Hence the part played by these latter (protein-sodium compounds), in determining the equilibria and maintaining the neutrality within the blood must be quantitatively comparable with that played by the bicarbonate." In Robertson's argument this conclusion results, so far as I can see, from his perfectly sound deduction that the sodium-protein

[1] T. Brailsford Robertson: this *Journal*, vi, p. 313, 1909.

[2] For a summary, see Henderson, *Ergebnisse der Physiologie*, viii, pp. 254–325, 1909.

[3] Henderson: *Amer. Journ of Physiol.*, xxi, p. 170, 1908.

compounds of blood are greater in amount than I had estimated them to be, as above reported. The question of course is strictly a quantitative one, and there seem to be adequate data for its solution. These data indicate that sodium-protein compounds are not more than one-fifth as useful as bicarbonates in the preservation of the neutrality of blood.

According to Szili[1] the average hydrogen ion concentration of normal blood at 18° is 0.30×10^{-7} and according to Benedict,[2] whose results were obtained in the same laboratory with the same apparatus, the hydrogen ion concentration of blood from diabetic coma (average of three cases) at 18° is $0.86 \times 10.^{-7}$ According to Bohr[3] the absorption coefficient of carbon dioxide in plasma at 38° is 0.541, and, according to the same author,[4] the average tension of carbon dioxide in blood may be taken as about 30 mm. I have shown[5] that in blood as the temperature rises from 18° to 38° the hydrogen ion concentration increases about 25 per cent. The ionization constant of carbonic acid at 18° according to Walker[6] is 3×10^{-7}, and according to Abbott[7] that of the ion $\overline{H_2PO_4}$ is 2×10^{-7}. At 38° these have risen to 4.2×10^{-7} (at least) and 2.4×10^{-7} respectively. For purposes of rough computation the degree of ionization (γ) of monosodium phosphate and sodium bicarbonate in blood may be taken to be about three-fourths.

The concentration law equation for the ionization of carbonic acid is

$$k \times (H_2CO_3) = (\overset{+}{H}) \times (\overset{-}{HCO_3})$$

and this may be converted into the equation[8]

$$(\overset{+}{H}) = \frac{k}{\gamma} \times \frac{H_2\,CO_3}{Na\;HCO_3}$$

[1] Szili: Plflüger's *Archiv*, cxv, p. 72, 1906.
[2] Benedict: Pflüger's *Archiv*, cxv, p. 106, 1906.
[3] Bohr: Nagel's *Handbuch*, i, (1), p. 63.
[4] Bohr: *ibid.*, p. 107.
[5] Henderson: *Amer. Journ. of Physiol.*, xxi, p. 427, 1908.
[6] Walker and Carmack: *Proc. Chem. Soc.*, xv, p. 208, 1899.
[7] See Henderson: *Loc. cit.*
[8] See *Ergebnisse der Physiologie*, viii, pp. 254–325, 1909.

Substituting in this equation data above given one obtains the following equation

$$\overset{+}{(\mathrm{H})} = \frac{4.2 \times 10^{-7} \times 4 \times 3 \times 0.541 \times 1.98}{3 \times 76 \times 44 \times \mathrm{Na\,HCO_3}}$$

which simplified yields the relationship

$$\mathrm{Na\,H\,CO_3} = \frac{5.4 \times 10^{-10}}{\overset{+}{(\mathrm{H})}}$$

For our present consideration it is a matter of little moment whether the above mentioned measurements of Szili and Benedict turn out to be strictly accurate or not, because no one can doubt that the hydrogen ion concentration of normal blood is somewhere near that determined by Szili, and countless observations indicate that blood hardly ever passes neutrality in its reaction; never until after long continued acid intoxication, or the feeding of large quantities of acid. Accordingly except in very extreme cases, if at all, we have to deal only with variations in reaction comparable with that chosen above as a fair case. What then are the ranges of variation in sodium bicarbonate and sodium protein between the reaction of normal blood $\overset{+}{(\mathrm{H})}_{33°} = 0.37 \times 10^{-7}$ and $\overset{+}{(\mathrm{H})}_{33°} = 1.00 \times 10^{-7}$ which corresponds to precise neutrality at 18°? This question answered, we shall possess a sound estimate of the relative importance of bicarbonates and sodium-protein compounds in neutralizing acid in the blood.

Substituting in the above equation these values for hydrogen ion concentration it appears that the concentration of sodium bicarbonate in normal blood is 0.0146N and in the second case it is 0.0054N, upon the assumption that in both cases carbonic acid tension is 30 mm. In this range bicarbonate has, then, neutralized an amount of acid equal to the difference between the these two quantities, that is to say, an amount of acid equal in volume to the blood, and about 0.01N. The measurements of sodium-protein combination which I have previously made[1] indicated that the variation in sodium-protein compounds for this

[1] Henderson: *Amer. Journ. of Physiol.*, xxi, p. 170, 1908.

same range is in the neighborhood of 0.001N, and I have now repeated these experiments, using neutral red as indicator and seeking to determine directly the amount of sodium required to transfer the proteins of blood at exact neutrality to the reaction of normal blood. The results are difficult to obtain because of the difficulty of using the indicator in the presence of proteins, still, so far as they go, they confirm the above conclusion, and the neutralizing power of the proteins of blood plasma between these two hydrogen ion concentrations seems to be not far from 0.001N, and certainly not more than 0.002N.[1]

It goes without saying that such measurements upon the proteins are open to doubt. One is dealing with substances more or less in true solution, more or less in colloidal suspension. Their actual state is indeterminate, and it is probably highly variable.[2] Moreover the unreliability of indicators in such instances, due probably to adsorption, is well known. Still there is little or no reason to doubt the order of magnitude of results obtained in this way. The difficulty is of course that we possess no means of obtaining perfectly unexceptionable results regarding the proteins by any method. The above estimate of the neutralizing power of proteins corresponds well on the whole with the known strength of amino-acids, and it would be difficult indeed in view of the known avidity of amino-acids, both in their basic and their acid characteristics, to account for a larger neutralizing power of proteins in true solution, unless the proteins were made up exclusively of the more strongly acid protein derivatives like aspartic and glutamic acids, or of some other acid substances, as in the case of casein. In basing some of his conclusions (pp. 313, 315, 316, 317, 318) upon the behavior of casein Robertson is in fact assuming a burden of proof, for, without evidence to the contrary, our chemical information would be taken as sufficient indication that casein is a far more acid substance than are the proteins of blood serum. But even casein, according to Robertson's data, seems to be far less efficient than the bicarbonate of blood to preserve neutrality through the range of reaction which we are here considering.

[1] I refrain from reporting results of titration which merely serve to confirm previous work.

[2] In such a case both the concentration law and the distribution law are of course involved.

I conclude then that in neutralizing acid, sodium-protein compounds of blood plasma in true solution are certainly not more than one-fifth as efficient as bicarbonates, unless existing data are seriously at fault. This conclusion rests upon the concentration law and upon no other foundation save experimental data. It differs from Robertson's conclusion because he, throughout his paper, is discussing variations in hydrogen ion concentration far greater than those which occur either normally or pathologically in the organism. It differs from Robertson's conclusion, in the second place, because he has nowhere estimated the magnitude of the simultaneous variations in bicarbonates, phosphates, and sodium-protein compounds which are indissolubly linked with variations in hydrogen ion concentration. Upon such a foundation alone can there be based an estimate of the relative importance of these several substances in neutrality regulation.

Within the cell phosphates[1] come to the aid of carbonates. The efficiency of phosphates in the muscle, for instance, is easily shown by a calculation analogous to that for carbonic acid to be about as great as that of bicarbonate, and the salts of muscle have a physiological neutralizing power approximately equivalent to a 0.02N solution of alkali. There is no reason to suppose that the increased concentration of proteins within the cell produces an effect comparable with this.

At p. 318 Robertson says: "Moreover after the total neutralization of the sodium in the blood by acid, carbonic and phosphoric acids can no longer protect the blood from increase in acidity, but the proteins can still neutralize a considerable quantity of acid." This consideration does not here concern us, because, long before any such event can occur, death must ensue, as clinical experience and experiment amply testify.

At p. 319 Robertson says: "Hence the quantity of protein in the blood is quite sufficient to account for all of the difference in CO_2-content between the arterial and venous blood." This conclusion is based upon the fact that sodium-protein compounds, as he estimates them, and variation in carbonic acid content from

[1] I am not aware that I have at any time suggested that phosphates are of importance in preserving neutrality in blood plasma.

arterial to venous blood are more or less equivalent. But the fact that they are equivalent is of no avail in arguing toward his conclusion. The question is whether the *variation* in sodium protein compounds in the blood is equivalent to the variation in carbonic acid as the blood flows through the capillaries of the lung. That seems to be highly improbable, and at any rate it can be established only by quantitative data bearing upon the point. One thing is certain, this carbonic acid excretion is associated with the movement of chlorine in and out of the blood cells as carbonic acid tension varies, according to the scheme which follows:

Lung or Tissue *Plasma* *Red Cell*

$$
\begin{array}{l|lcr|l}
CO_2 \rightleftarrows & CO_2 + H_2O \rightleftarrows H_2CO_3 & & \overset{+}{H} + \overset{-}{Cl} \rightleftarrows & \overset{-}{Cl} \\
 & \qquad\qquad\qquad\quad + & & \upharpoonleft\downharpoonright \quad & \\
 & \qquad\qquad NaCl \rightleftarrows NaHCO_3 + HCl & & &
\end{array}
$$

This necessary consequence of the well known phenomenon of Zuntz[1] has so far as I am aware not been pointed out in this connection. Certain it is that we have as yet no proved theory concerning the mechanism of carbonic acid removal from the blood, so far as the chemical equilibria are concerned.

At p. 317 Robertson speaks of the occurrence of carbonates, as well as of bicarbonates in the blood. But carbonates cannot exist there in more than infinitesimal amounts, their concentration being little more than that of the hydroxyl ion, and they take no part in this, or, so far as is known, in any other physiological process.

SUMMARY.

(1) (Cf. Robertson, p. 319.) The power of proteins to preserve neutrality in solution, though great in itself, is, under conditions which obtain in the organism, not comparable with that of the inorganic constituents of blood and protoplasm.

(2) (Cf. Robertson, pp. 319, 320.) There is no proof that more than a small portion of the carbon dioxide liberated from the blood in the lungs comes from sodium bicarbonate which

[1] See for literature, etc., Spiro and Henderson, *Biochemische Zeitschrift*, xv, p. 114, 1908.

has given up its sodium to proteins, according to a simple reaction which is reversed in the tissues. It is extremely improbable, whatever else may explain the process, that this is the chief reaction involved, unless indirectly through the heterogeneous equilibrium between red cells and plasma, including the movement of chlorine, ionized or otherwise, across the red cell wall, and the consequent liberation and fixation of carbonic acid in the plasma.

OBSERVATIONS ON THE INFLUENCE OF LACTIC ACID FERMENTS UPON INTESTINAL PUTREFACTION IN A HEALTHY INDIVIDUAL.

By HELEN BALDWIN.

(*From the Laboratory of Dr. C. A. Herter, New York.*)

(Received for publication, October 28, 1909.)

Of all forms of bacterial invasion into the human body, there are few more difficult to reach and to combat than those of putrefactive bacteria in the intestinal tract. Since the more virulent of such organisms, colonizing in the intestines, have their development retarded or inhibited in an acid medium, an effort is being made by Metchnikoff and others to treat intestinal putrefaction by introducing into the stomach living cultures of lactic acid organisms, in the hope that these by continuing to grow in the intestine may there produce an acid reaction and so prevent putrefactive decomposition of the contents of the intestine. It has been proved by Metchnikoff[1] and Cohendy.[2] in the case of men, and by Herter and Kendall,[3] in the case of monkeys, that certain of the lactic acid producing organisms, as *B. bulgaricus* retain their vitality while passing through the digestive tract and that when they are given by mouth, they can be grown in cultures from the feces. The effect upon intestinal putrefaction of such ingestion of lactic acid ferments can be discovered only by a large number of observations under normal and pathological conditions.

The following report is a contribution to such a body of evidence. It is a study of the effect of the ingestion of certain lactic acid ferments upon intestinal putrefaction when they are given in combination with an ordinary mixed diet. The subject of the record was a healthy man, 43 years old, who throughout

[1] Metchnikoff: *The Prolongation of Life.*
[2] Cohendy: *Compt. rend. de la soc. de biol.*, lx, 1906.
[3] Herter and Kendall: This *Journal*, v, p. 293, 1908–1909.

the period of the experiment led a life of ordinary routine with regular hours of work, of exercise and of sleep. The observations extend over a continuous period of four months, during which time lactobacilline malt, bacillac, zoolak and plain milk were in turn taken in addition to an ordinary diet. The purpose of the experiment was to watch under these varying conditions, the development of putrefactive products in the intestines and the loss of nitrogen in the feces.

Throughout the period of experiment daily examinations of the urine and feces were made, occasionally two days specimens or urine being combined and examined together. The quantitative analyses of the urine and feces were made by Mr. Edward N. O'Brien. Throughout the most of the period the quantitative estimations were all done in duplicate, thus increasing the accuracy of the results. The following points were daily determined in the urine: the volume, color, reaction, specific gravity, the presence of indol and indolacetic acid, the reaction in the acid distillate with Millon's reagent, and the determination of the total nitrogen, the inorganic sulphates and the ethereal sulphates. In the feces, the total nitrogen, the percentage of water and the total amount of fresh and dried feces were determined.[1] Also there were noted the color, consistency, reaction and Schmidt's test for hydrobilirubin. A portion of the feces was rendered acid with phosphoric acid and distilled. This distillate was tested with Millon's reagent and with the *p*-dimethylamino-benzaldehyde reagent. In Table I are given the average results of the quantitative determinations made during the different periods.

A series of observations had been made upon the same man during the summer of 1908. The record of his normal condition at that time was used for comparison with the observations noted in the present experiment. Including these former notes, the dietary periods of this series were the following:

[1] *Methods.* The total nitrogen of the urine and of the feces was determined by the Kjeldahl method.

The inorganic and ethereal sulphates were determined by Folin's method (this *Journal* i, p. 131, 1906).

The indican was followed by means of Folin's method (*American Journal of Physiology*, xiii, p. 705).

TABLE 1

PERIOD	DIET AND LACTIC ACID FERMENT TAKEN		PREFORMED SULPHATES (H_2SO_4)	COMBINED SULPHATES (ETHEREAL) H_2SO_4	RATIO. $\frac{\text{PREFORMED SULPHATES}}{\text{ETHEREAL SULPHATES}}$	TOTAL NITROGEN OF URINE	WEIGHT—FRESH FECES	WEIGHT—DRY FECES	PERCENTAGE OF WATER IN FECES	NITROGEN OF FECES	NITROGEN OF URINE + FECES
I	Mixed diet only	Daily average for 28 days	2.233	0.216	10.5	12.9	97.6	19.2	80.3	1.04	13.9
II	Mixed diet	2 "	2.187	0.179	12.2	12.1	65.8	16.7	74.6	1.16	13.2
III	Mixed diet: Lactobacilline tablets	3 "	2.721	0.292	9.0	14.8	132.3	25.9	80.4	1.73	16.6
IV	Mixed diet: Lactobacilline malt 50cc. twice daily	first 22 days	2.108	0.261	8.1	13.2	90.7	21.5	76.3	1.40	14.6
		second 22 days	2.072	0.254	8.2	12.6	96.0	21.8	76.2	1.41	14.0
V	Milk and biscuits only	7 days	2.448	0.150	16.3	14.7	124.3	25.4	79.7	0.89	15.5
VI	Mixed diet, breakfast and dinner. Plain milk and biscuits, lunch	10 "	2.183	0.251	8.7	12.9	115.0	28.3	75.4	1.23	14.1
VII	Mixed diet, breakfast and dinner. Bacillac and biscuits, lunch	10 "	2.456	0.263	9.3	14.2	102.0	25.2	66.8	1.39	15.5
VIII	Mixed diet, breakfast and dinner. Plain milk and biscuits, lunch	10 "	2.424	0.267	9.0	14.2	128.6	29.4	67.7	1.41	15.6
IX	Mixed diet, breakfast and dinner. Zoolak and biscuits, lunch	10 "	2.375	0.187	12.8	13.9	120.7	27.4	77.3	1.40	15.3
X	Mixed diet only	15 "	2.409	0.180	13.4	14.3	122.7	23.7	80.0	1.53	15.9
XI	Mixed diet, breakfast, lunch and dinner. Zoolak, one liter daily	7 "	2.516	0.239	10.5	14.0	129.2	27.9	78.4	1.63	15.7

I. Ordinary mixed diet: 28 days, May 29 to June 26, 1908.
II. Ordinary mixed diet: 2 days, December 8 and 9.
III. One lactobacilline tablet after each meal. Ordinary mixed diet: 3 days only,[1] December 10 to 12.
IV. Lactobacilline malt, 50 cc. twice daily before meals. Ordinary mixed diet: 44 days, December 13 to January 25.
V. Plain milk and biscuits only: 7 days, January 26 to February 1.
VI. One liter of plain milk and 90 grams of biscuits only for lunch. Ordinary mixed diet for breakfast and dinner: 10 days, February 3 to 12.
VII. Bacillac, one liter and biscuits, 90 grams for lunch. Ordinary mixed diet for breakfast and dinner: 10 days, February 13 to 22.
VIII. Plain milk, one liter, biscuits, 90 grams for lunch. Ordinary mixed diet for breakfast and dinner: 10 days, February 23 to March 4.
IX. Zoolak, one liter and biscuits, 90 grams for lunch. Ordinary mixed diet for breakfast and dinner: 10 days, March 4 to 15.
X. Ordinary mixed diet: 15 days, March 15 to 29.
XI. Zoolak, one liter daily in addition to ordinary mixed diet three times daily: 7 days, March 30 to April 6.

There were no symptoms produced by the varying diets excepting that twice during the period when lactobacilline malt was taken the eating of an apple was followed by a severe attack of colic with a slight diarrhœa.

The variations in the amounts of indol and phenol and their derivatives excreted from the body in the urine and feces are used as an approximate index of the degree of intestinal putrefaction since indol and phenol are not formed in the body in appreciable quantities excepting through the activity of putrefactive bacteria and barring some especial focus elsewhere, only in the intestines. The amount of indican and phenol and allied substances excreted cannot however be taken as an absolute index of that formed in the intestine as there may be some degree of putrefaction in the food brought before it is ingested and there may be putrefactive changes in the food in the stomach if there is an absence of hydrochloric acid in the gastric contents.

[1] The period of three days when lactobacilline tablets were given is included in the record as it formed a part of the period of experiment but no conclusions are drawn from the observations at that time because the period was of such short duration.

Ethereal Sulphates. Daily estimations of the ethereal sulphates in the urine were made because the indol, phenol, skatol and allied putrefactive products which are absorbed from the intestines become linked with sulphuric acid in the body and are excreted as ethereal (or combined) sulphates. In health the amounts of the ethereal sulphates vary regularly with the amount of proteid ingested and bear an approximately constant ratio to the nitrogen excreted in the urine. (The ratio of the amount of ethereal sulphates excreted in health, measured as sulphuric acid, to the total nitrogen of the urine is as 1.4 to 1.5 : 100 "coefficient of Amann.")

The accompanying charts show the relative amounts of nitrogen and ethereal sulphates (estimated as H_2SO_4) excreted daily in the urine. Chart I gives the absolute daily amounts throughout the experiment. Chart II gives the average daily amounts for the different periods, thus showing more clearly the variations due to the different diets. The ordinates for the nitrogen curves and the sulphuric acid curves bear the ratio of 100 to 1.5 so that in health the two curves should approximately coincide.

Apart from the plain milk period the intestinal putrefaction, as measured by the ratio of the ethereal sulphates to the nitrogen in the urine was least when an ordinary mixed diet was given with no additional fermented milk or malt. This is seen in the period of one month in June 1908 (Period I), in the period of two days only just before the beginning of the experiment (Period II) and in the period of sixteen days which came almost at the end of the experiment (Period X).

In the period when zoolak and biscuits only were taken for lunch with a mixed diet for breakfast and dinner (Period IX) the ethereal sulphates became less than normal but in the last period when a liter of zoolak was taken daily in addition to an ordinary diet for lunch as well as breakfast and dinner (Period XI) the intestinal putrefaction again increased over that of the period before when no fermented milk was taken. In both these zoolak periods the ratio of ethereal sulphates was less than during the time when other forms of the products of lactic acid fermentation were given. The intestinal putrefaction as measured by the ratio of the ethereal sulphates was highest dur-

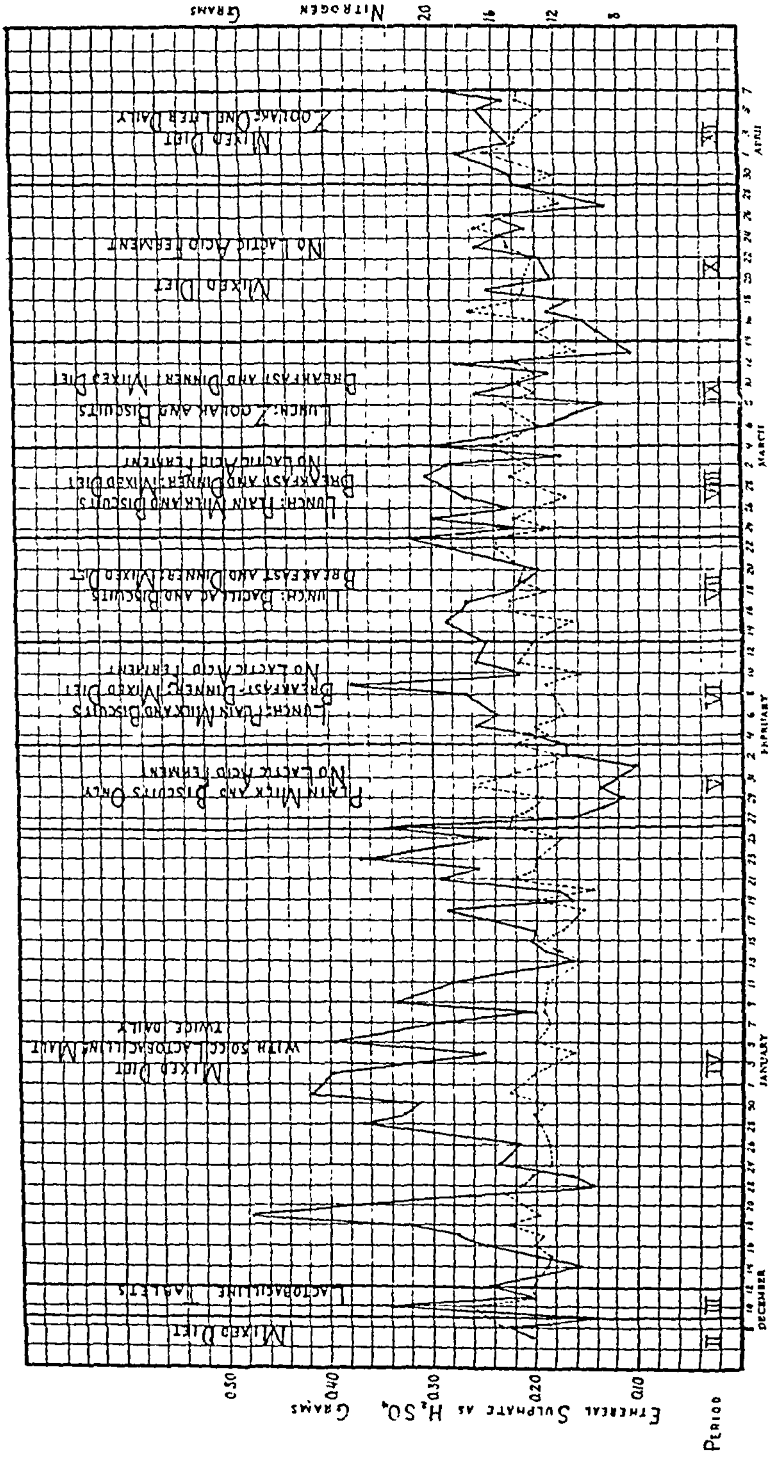

CHART I. Showing daily amounts of ethereal sulphates as H_2SO_4 (solid line) and of nitrogen (broken line) excreted in the urine.

The scales of ordinates are so arranged that the two curves should normally coincide.

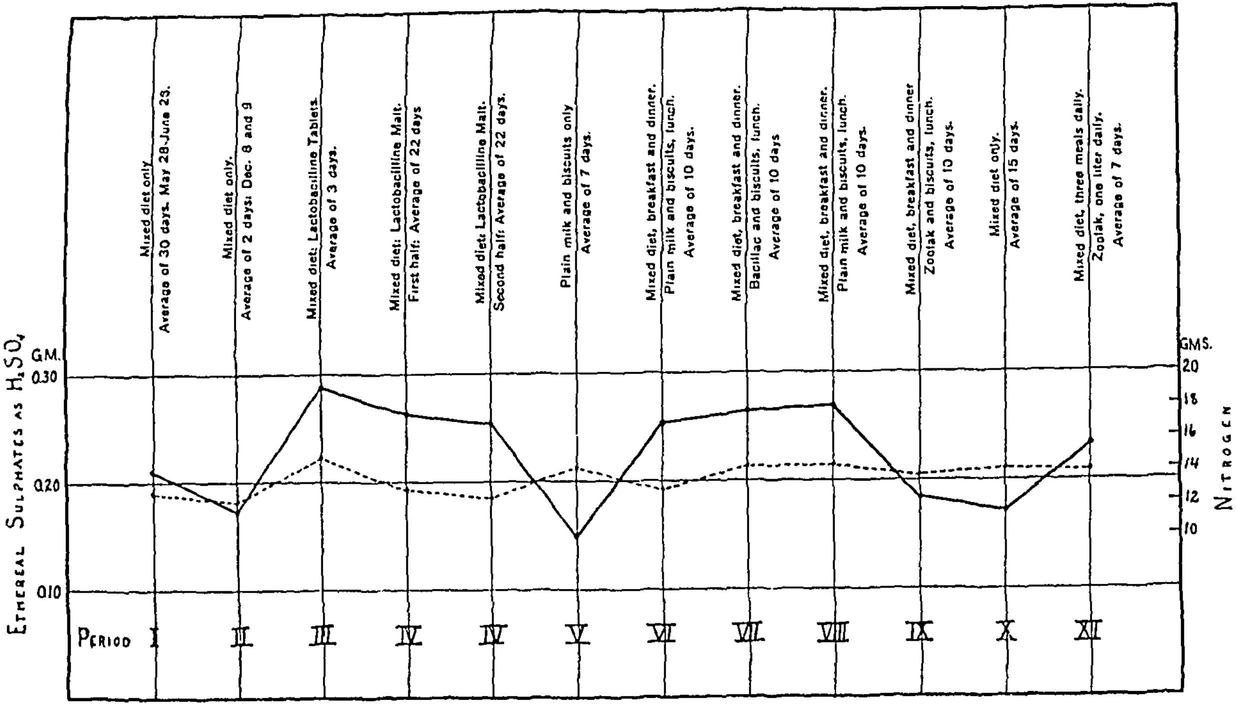

Chart II. Showing *average* daily amounts of ethereal sulphates as H_2SO_4 (solid line) and of nitrogen (broken line) excreted in the urine during the different periods.

The scales of ordinates are so arranged that the two curves should normally coincide.

ing the period when lactobacilline malt was given (Period IV) but it was nearly as high during the periods when bacillac was given (Period VII) and when a liter of plain milk with biscuits only was taken for lunch with an ordinary mixed diet for breakfast and dinner (Periods VI and VIII).

The week when plain milk and biscuits only were taken forms a break between the lactobacilline malt period and that which followed, showing that the influence of the lactobacilline malt was not carried over to the later periods. The ethereal sulphates are always markedly reduced when a patient is put on a milk diet, as is shown in the charts (Period V).

Indican. The examination made the first day of the experiment when a general mixed diet was taken showed a trace of indican in the urine. The next day on the same diet there was none.

Throughout the various periods there was but little difference in the amount of indican found, usually there was none, often a trace only, seldom more than a trace. During the week when milk and biscuits only were taken, the indican was somewhat increased. Also when a liter of plain milk was taken at noon with a mixed diet for breakfast and dinner the amount of indican was more than during the other periods, at one time giving a marked reaction (75 in a color scale in which Fehling's Solution equals 100).

Indolacetic Acid. The urine throughout the experiment showed only traces of indolacetic acid excepting during the period when lactobacilline malt was given. At that time the reaction became more marked.

Phenols. While indol and the phenols are both regularly formed by the putrefactive processes in the intestine, there is no definite parallelism between the amounts of the phenol and indican found in the urine. This is due to variation always occurring in the amounts formed, the amounts absorbed from the intestine and the amount of indol oxidized in the body.

The phenol and allied substances reacting with Millon's reagent in the urinary distillate were found only in small amounts throughout the period of experiment although occasionally there was a marked reaction for these bodies. The average amount of phenol was greatest during the period when lactobacilline malt

was taken and in the period when a general mixed diet alone was taken. It was least in the period when zoolak and biscuits only were taken for lunch and the amount was exceptionally low when plain milk and when bacillac with biscuits only were taken for lunch.

Indol and Skatol in the Feces. The acid distillate from the feces was tested daily with the *p*-dimethylamino-benzaldehyde reagent. This serves as a delicate test for skatol and indol, giving a rosy or red color with indol, and a violaceous or purple color with skatol. Other substances react with this reagent but in the acid distillate from the feces these products of putrefaction, indol and skatol, seem to be dominant in giving the reaction. The average intensity of this reaction was least in the two periods when zoolak was given. It was nearly as low in the periods when an ordinary mixed diet was given and in one of the periods when plain milk and biscuits were given for lunch in addition to an ordinary mixed diet for breakfast and dinner. It was most marked during the periods when bacillac, lactobacilline malt and lactobacilline tablets were given.

Phenols in the Feces. The phenols are so readily absorbed from the intestine that only small amounts are ordinarily found in the feces. Throughout this series of observations, the acid distillate from the feces was tested with Millon's reagent but the amount of phenols present was always too small to be detected by the method used.

Reaction of the Feces. The reaction of the feces was alkaline throughout the period of experiment, excepting for three days. Twice during the period when the subject was on an ordinary mixed diet, the feces became acid and one day during the last period, when zoolak was added to a full mixed diet, the reaction was slightly acid. These changes to an acid reaction were associated with a tendency to diarrhœa. That the reaction of the feces was alkaline during the periods when the fermented milk and malt were taken, showed that all the acid produced by the lactic acid organisms had been neutralized or absorbed from the intestines before the feces were voided. This result is in contrast to the observations of Grekoff,[1] who found in patients suf-

[1] Grekoff: *Observations clinique sur l'effet du lait aigri de Metchnikoff dans les maladies intestinales.* St. Pétersbourg, 1907.

fering from intestinal affections, that when the cases resulted favorably the fecal matter gave an acid reaction after the fourth or fifth day of treatment with lactic acid preparations.

Schmidt's Reaction in the Feces. When a concentrated solution of mercuric chloride is thoroughly mixed with the feces a marked red color with yellowish fluorescence may develop. This has been regarded as a test for hydrobilirubin. It has been found more marked in cases of intestinal putrefaction and has been thought to indicate an increase in reduction processes in the intestine due to the excessive action of putrefactive bacteria. Throughout the period of experiment daily tests were made for Schmidt's reaction in the feces. This reaction was negative or very slight during the lactobacilline malt period and it was slight throughout the most of the period when bacillac was given. The reaction was marked throughout all periods when plain milk was given, either with biscuits only or in addition to a mixed diet. There was a moderate reaction during the periods when an ordinary diet of zoolak added to an ordinary diet were given.

Lactic Acid Bacilli in the Feces. At the close of the lactobacilline malt period and again a week after the lactobacilline malt was discontinued, the coarse lactic acid bacilli in the lactobacilline malt (*B. bulgaricus?*) were grown in cultures from the feces by Dr. A. I. Kendall.

In reviewing the record of this case, one has to recognize the narrow limits of the experiment. The observations were made upon only one person and he was in health. During each period an ordinary mixed diet was taken together with the fermented milk or malt. The observations with regard to each form of lactic acid ferment were continued for only ten days in succession with the exception of lactobacilline malt when the record covered forty-four successive days. The variations from the normal, as would be expected, were only slight. No generalizations can be made from a single case. Individual idiosyncracies of digestion are so marked that the saying is constantly being proved that what is one man's meat is another man's poison. The results as they stand are interesting, however, especially as showing that in this case the subject's condition, as indicated by the ratio of ethereal sulphates excreted, was best when he was taking an ordinary mixed diet of his own choosing, with no form of lactic acid

ferment or of milk food excepting the small amount of milk added to the tea and coffee. The excretion of phenol in the urine was slightly greater during the period when a mixed diet alone was taken but this was the only respect in which the subject's condition was not fully as favorable or even more favorable than during the other periods.

During the period when zoolak and biscuits only were taken for lunch, there were days when the ratio of the ethereal sulphates was very low, lower than at any other time excepting when biscuits and milk were the only foods taken. This is best shown in Chart I. The amount of phenol excreted in the urine was also the lowest during the period when zoolak and biscuits only were taken for lunch. But in the last period when a liter of zoolak was taken in addition to three meals daily of a mixed diet the subject's condition again became less satisfactory than when no fermented milk was added to the diet.

Lactobacilline malt was given continuously for so long a period (44 days) that its effect can be more definitely stated than that of the milk preparations. The period was divided into two parts to determine whether the condition varied in the second half from the first half of the time. The averages for these two halves of the period vary but slightly in any respect as can be seen by the table of averages of the periods (Table I). The subject's condition was definitely less satisfactory during the lactobacilline malt period than at any other time. During this period the ratio of ethereal sulphates was greater, the excretion of phenol was greater, the reaction for indol and skatol in the acid distillate from the feces was more marked than during any other period. Also, the percentage of nitrogen lost in the feces during the second half of the period was greater than at any other time excepting the last period when zoolak was taken in addition to a full diet. Twice during this period there occurred attacks of severe colic when apples were eaten, the combination of the lactobacilline malt and the fruit apparently causing the colic. There was more constipation during this period. Throughout the other periods the bowels were fairly regular. The amount of fresh feces and of dried feces was least during the lactobacilline malt period. This was evidently due to the fact that the lactobacilline malt lessened the appetite for other food as the nitrogen in

the urine was also low at this time, showing that there was a lessening and not an increase in the amount of nitrogenous food absorbed from the intestine. Schmidt's reaction in the feces was negative throughout the lactobacilline malt period. If, as is supposed, this indicates diminished reduction processes in the intestines due to a lessening of the activity of putrefactive bacteria, the patient's condition in this regard was better than at any time during the experiment.

The periods in which a liter of plain milk with biscuits was taken for lunch with a mixed diet for breakfast and dinner showed a definite increase in intestinal putrefaction over the periods when a mixed diet with only the small amount of milk added to the tea and coffee was taken three times in a day. When milk and meat were both taken the ethereal sulphates were increased (Charts I and II, Periods VI and VIII), the indican was increased, Schmidt's reaction in the feces was more marked; but the reaction for phenol in the urine was slight during these periods.

Emphasis should be laid upon the fact that the observations in this paper have no bearing upon the use of fermented milks alone or with a diet free from meat. The giving of protein in the form of milk rather than of meat regularly reduces the amount of intestinal putrefaction. The fermented milks give a variety to a milk diet, they are found by many to be more appetizing and refreshing than plain milk and in certain forms of gastric and intestinal indigestion they are better borne than plain milk. The object of this investigation as before stated was only to determine the effect of fermented milks in controlling intestinal putrefaction when a general mixed diet including meat was taken. *In this case it was found that they exerted no specially favorable* influence in this way but in so far as the subject's condition varied it was better when the lactic acid ferments were not added to the general diet.

In conclusion I am glad to acknowledge my indebtedness to Mr. E. N. O'Brien for his coöperation in performing a large part of the analytical work connected with this study.

THE CATALYTIC ACTION OF AMINO-ACIDS, PEPTONES AND PROTEINS IN EFFECTING CERTAIN SYNTHESES.

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(From the Laboratory of Dr. C. A. Herter, New York.)

(Received for publication, October 5, 1909.)

Our knowledge of the synthetic reactions concerned in the formation of fats or carbohydrates which must play so important a rôle in cell metabolism is meager in the extreme but it is profitable to try and compare in a crude fashion certain known types of chemical change with the more complicated biochemical transformations, in the hope of discovering analogies between the two sets of phenomena.

It is natural to suppose that biochemical syntheses are controlled by catalytic agents as is the case with the biochemical oxidations and hydrolyses, but of the nature of these catalysts practically nothing is known. In the following paper it will be shown that certain syntheses may be effected by the use of the amino-acids, peptones, etc., as catalysts. The reactions to be described are not known, in most cases, to normally occur in the living cell but it is possible that they represent types of reactions which actually do occur.

In considering, from a purely chemical standpoint, the possible nature of a biochemical synthesis of a fatty acid, it will be found that there are a few well-defined chemical reactions which present certain analogies. These reactions are of the type in which an aldehyde, R.CHO, undergoes condensation with another aldehyde molecule, or with a negatively substituted acetic acid derivative.

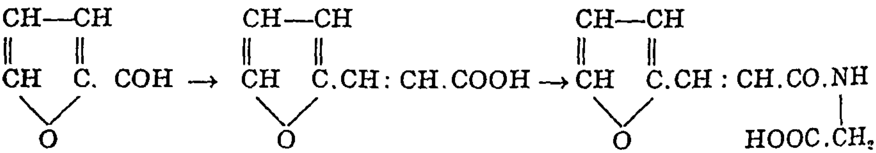
That reactions of this type may occur to the animal organism is shown by Friedmann's demonstration of the formation of acetone from acetaldehyde when added to blood perfused through a surviving liver. The aldehyde undergoes the aldol

condensation, the product being oxidized to β-oxybutyric acid which is then further oxidized by an enzyme in the liver to form acetone through the intermediate stage of aceto-acetic acid.

$$2CH_3.C\begin{matrix}\diagup O\\ \diagdown H\end{matrix} \rightarrow CH_3.CHOH.CH_2.C\begin{matrix}\diagup O\\ \diagdown H\end{matrix} \rightarrow CH_3.CHOH.CH_2.COOH$$

The probability that this type of change, namely, the aldol condensation, would prove to be of biochemical significance had already indicated by Magnus-Levy, Leathes and Raper.[1]

Another well defined type of biochemical reaction concerns the condensation of aldehydes and acids. The remarkable observation has been made that when furfurol is administered subcutaneously to a dog, a part of the substance is excreted in the form of the glycocoll derivative of furfuracrylic acid.[2]



The first steps in this remarkable synthesis, namely, the formation of furfuracrylic acid is of the same type as the well known Perkin reaction by which, for example, cinnamic acid is obtained from benzaldehyde. It is clear, therefore, that in order to obtain purely chemical analogies for some of the synthetic reactions of the living cell, it will be well to pay special attention to condensations of the type just indicated.

The conditions under which the Perkin and many other related condensations are usually carried out, are those of high temperature, absence of water and the use of reagents such as acetic or other acid anhydride. These conditions are obviously far removed from those which could possibly prevail in the living cell. It has, however, been found by Knoevenagel that an enormous number of related syntheses between aldehydes and ketones of various kinds on the one hand and a variety of substances containing the $-CO.CH_2.CO-$ or related group on

[1] Cp. Leathes: *Ergebnisse der Physiologie*, p. 356, 1909.
[2] Jaffé and Cohn: *Ber. d. deutsch. chem. Gessellsch.*, xxx, p. 2315.

the other, may be effected at low temperatures and without the use of dehydrating agents, by the employment of organic bases such as diethylamine, piperidine, aniline, etc., as catalysts.[1]

The catalytic action of these bases in effecting condensation is by Knoevenagel believed to be due to the formation of a reactive addition compound between the aldehyde and base, which then undergoes condensation with the third substance with regeneration of the base. Hann and Lapworth[2] advance another explanation based upon the changes in ionization brought about by the addition of the strong base.

These striking investigations of Knoevenagel's form an attractive basis for speculation as to whether similar reactions may not occur in the living cell and Schryver has already made some experiments in this connection but with negative results.[3]

The object of the following communication is to drawn attention to the fact that many of these condensations, which may possibly be analogous to some of those occurring in the living cell, may be brought about by the use of amino-acids, peptones, albumoses and even proteins as catalysts.

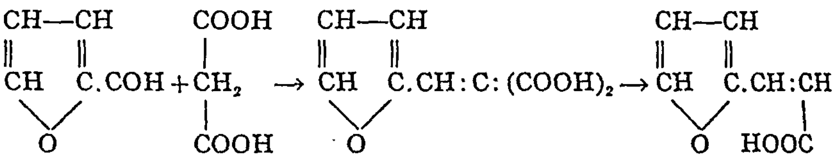
For example, furfurol when warmed with malonic acid does not undergo condensation, but if a simple amino-acid, such as glycocoll or alanine, be added condensation takes place with the formation of considerable amounts of furfuracrylic acid. As already mentioned an identical conversion of furfurol into furfuracrylic acid takes place in the body, although of course it is an open question as to how far the syntheses are similar in detail.

If instead of using malonic acid, sodium malonate be employed condensation may be effected at 37° by means of glycocoll, alanine, peptone, etc., with formation of furalmalonic acid, which on heating loses carbon dioxide and yields furfuracrylic acid as before:

[1] Many papers in *Berichte* during last ten years.

[2] *Trans. Chem. Soc.*, lxxxv, p. 46.

[3] *Journ. of Physiol.* xxx, *Proc. Physiol. Soc.*, Jan., 1904.



(furalmalonic acid) (furfuracrylic acid)

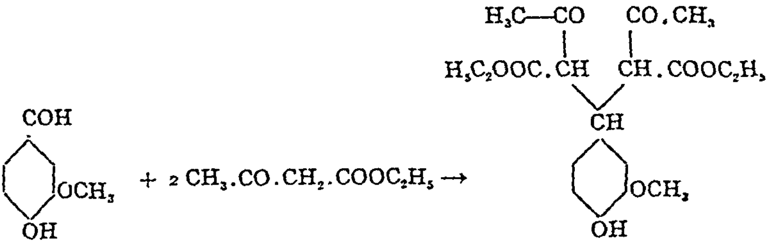
A number of similar condensations have been effected using amino-acids or related substances as catalysts. For example, benzaldehyde may undergo condensation with malonic acid or sodium malonate with formation of cinnamic acid or benzalmalonic acid according to the conditions. A variety of amino-acids derived from proteins were successfully used for effecting this condensation while the following substances were found to be practically without favorable action: urea, acetamide, asparagine, uric acid, caffeine, hippuric acid, glucosamine. Ammonium carbonate brought about some condensation but its action was not so marked as in the case of the amino-acids.

The formation of the glycocoll derivative of cinnamic acid from benzaldehyde would represent the same type of synthesis as was observed by Jaffé and Cohn in the case of furfurol as already mentioned. Since the writer had actually isolated cinnamoylglycocoll from the urines of cats under certain circumstances,[1] it was clearly of interest to determine whether the substance could not be detected in the urines of animals that had benzaldehyde administered to them. Actual experiments with such large doses of benzaldehyde that the cats died after about twenty-four hours failed to reveal the presence of significant amount of cinnamic acid derivatives in the urine.

Cinnamic aldehyde is a particularly suitable aldehyde for demonstrating the catalytic action of the amino-acids in effecting condensations with malonic acid since the resulting cinnamylidene-malonic acid readily separates out from solution in the form of bright yellow crystals. A mixture of equal weights of cinnamic aldehyde and malonic acid dissolved in a little dilute alcohol may be digested at 37° for a long time without any separation of crystals. If, however, an amino-acid or peptone, etc., be added an excellent yield of the condensation product is readily obtained.

[1] This *Journal*, vi, p. 204.

Another type of reaction that was successfully effected was the condensation of an aldehyde (vanillin) with aceto-acetic ester by means of arginine carbonate. A good yield of vanillidene-di-aceto-acetate-ethyl-ester was readily obtained.



On the other hand attempts to bring about the following types of condensation were unsuccessful:

(1) Aldol condensation. Acetaldehyde was allowed to stand at temperatures varying from 0° to 28° mixed with strong aqueous solutions of glycocoll or alanine. No aldol could be detected although paraldehyde was formed.

(2) Condensation between aldehydes (e. g., benzaldehyde) with ketones (acetone). No action was observed.

(3) Condensation between benzaldehyde and acetaldehyde. No cinnamic aldehyde was formed using alanine as condensing agent.

(4) Benzaldehyde and hippuric acid could not be induced to undergo condensation by means of amino-acid.

The results of the investigation are contained in the following table. In general the experiments were made by dissolving the malonic acid (1 mol.) or other substance together with about half its weight of amino-acid in the minimum amount of water and then adding the aldehyde (1 mol.). In most cases it was necessary to add a few drops of alcohol to bring the aldehyde into solution. The tubes containing the mixture were then either incubated at 37° or heated in a water-bath at 100°. The details of the separation of the condensation products are omitted for the reason that that they were the ones usually employed for the purpose and because all of the substances are well known and easily characterized. In every case where a positive reaction is indicated in the table it is to be inferred that the product

was obtained in the pure crystalline state with correct melting point. A rough approximation of the yields is given in table. "Good" indicates over 50 per cent of the theoretical amount "fair" represents a yield of from 10 to 50 per cent, while "poor" describes the yields of less than 10 per cent.

REACTING SUBSTANCES	CATALYST	TEMP.	CONDENSATION	PRODUCT	YIELD
		degrees			
(1) Furfurol + malonic acid	glycocoll	100	+	Furfuracrylic acid	fair
(2) " "	alanine	100	+	"	fair
(3) Furfurol + sodium malonate		37	–		
(4) " "	alanine	37	+	furalmalonic acid	good
(5) " "	peptone	37	+	"	fair
(6) " "	albumose	37	+	"	poor
(7) Benzaldehyde + malonic acid		100	–		
(8) " "	glycocoll	100	+	cinnamic acid	fair
(9) " "	alanine	100	+	"	fair
(10) " "	leucine	100	+	"	fair
(11) Benzaldehyde + sodium malonate		100	–		
(12) " "	alanine	37	+	benzalmalonic acid	fair
(13) " "	"	100	+	benzalmalonic traces cinnamic	fair
(14) " "	peptone	100	+	"	fair
(15) o-Nitrobenzaldehyde + malonic acid	alanine	100	+	o-nitrocinnamic acid	good
(16) m-Nitrobenzaldehyde + malonic acid	glycocoll	100	+	m-nitrocinnamic acid	good
(17) p-Nitrobenzaldehyde + malonic acid	alanine	100	+	p-nitrocinnamic acid	good
(18) Cinnamic aldehyde + malonic acid		37	–		
(19) " "	glycocoll	37	+	cinnamylidene-malonic acid	good
(20) " "	alanine	37	+	"	good
(21) " "	leucine	37	+	"	good

REACTING SUBSTANCES	CATALYST	TEMP.	CONDENSATION	PRODUCT	YIELD
		degrees			
(22) Cinnamic aldehyde + malonic acid	aspartic acid	37	+	"	good but reaction slow
(23) " "	glutamic acid	37	+	"	"
(24) " "	arginine carbonate				good
(25) " "	tyrosine	37	+	"	poor
(26) " "	phenyl-alanine	37	+	"	poor
(27) " "	clupeine	37	+	"	poor
(29) " "	peptone	37	+	"	good
(30) " "	albumose	37	+	"	fair
(31) " "	egg-white	37	+	"	poor
(32) Vanillin + aceto-acetic ester	arginine carbonate	60	+	Vanillidene-di-aceto-acetic ethyl ester	good

NOTE ON THE UROROSEIN REACTION.

By H. D. DAKIN.

(*From the Laboratory of Dr. C. A. Herter, New York.*)

(Received for publication, October 5, 1909.)

In recent papers upon the action of hydroxylamine upon ketones of the type CHR : CH.CH : CH.COR Ciusa and Terni[1] have recently described a number of interesting derivatives of cinnamylideneacetophenone and incidentally they, with Luzetto, recorded the fate of some of these substances when administered to animals. The administration of one of these substances, the α-oxime, was followed by a marked urorosein reaction in the urine and they state that this is the more remarkable since this change had not previously been induced by artificial means.

Herter[2] has however shown that indolacetic acid is at least one, if not the only chromogen of urorosein and that this substance when accompanied in urine by nitrites, which may originate in various ways, will furnish a typical urorosein reaction.

As there was a remote possibility of Ciusa and Terni's compound undergoing intramolecular rearrangements in the organism with formation of indol derivatives, it was considered of interest to try and repeat their observations. It was found, however, that neither the α-oxime nor any of the other hydroxylamine derivatives of cinnamylideneacetophenone described by Ciusa and Terni, when administered either by mouth or subcutaneously to dogs or rabbits was capable of inducing the urorosein reaction. These results therefore do not harmonize with those of Cuisa and Terni.

It is suggested as a possibility that the urines of the animals employed by the latter investigators contained minute quanti-

[1] *Atti. R. Accad. Lincei.* [v] xv, ii, p. 455 and xvii, i, p, 724, 1908.

[2] This *Journal*, iv, p. 253.

ties of indolacetic acid and the administration of the oximes was followed by the occurrence of nitrites in the urine, for it is known that hydroxylamine is converted into nitrous acid in the organism. The simultaneous presence of these two substances would account for the observed urorosein reaction.

NOTES ON THE ACTION OF SODIUM BENZOATE ON THE MULTIPLICATION AND GAS PRODUCTION OF VARIOUS BACTERIA.

By C. A. HERTER.

(Received for publication October 12, 1909.)

In the course of the summer of 1908, while carrying on a research on the action of sodium benzoate on the human body, which has been published in Bulletin No. 88 of the United States Department of Agriculture, observations were also made upon the influence of sodium benzoate upon the multiplication and gas production of various kinds of bacteria, and some of these observations appear to be of sufficient interest to make it worth while to place them on record, especially in view of the interest that has been roused recently through the discussion of the influence of sodium benzoate upon the human organism. The observations upon the effect of sodium benzoate on the multiplication and gas production of bacteria, which form the subject matter of these notes, do not constitute a systematic examination of the subject in question, but they are sufficiently numerous and varied to establish some features of interest which I think have not been heretofore noted.

I. One portion of the work related to the examination of various canned and bottled foodstuffs and condiments. At my request, made through the secretary of the National Food Manufacturers' Association, thirty-seven different varieties of canned and bottled foods were sent to me for examination. This list does not include certain samples of codfish which had been spoiled through the action of bacteria, which were sent to me from Gloucester, Mass. The food-stuffs and condiments sent me included samples of chili sauce, catsup, sweet pickles; table syrup; peach, blueberry, strawberry, damson, blackberry, apricot, pineapple and white cherry preserves; strawberry, blackberry, apricot, pineapple and white cherry jam; currant

jelly; lemon and prune pie-filling; apple cider; and various soda fountain syrups.

The procedure followed in the examination of these foodstuffs was to introduce about 0.5 gram of the solid or semi-solid material into dextrose, lactose and saccharose bouillon which had been sterilized in fermentation tubes. In the case of fluid preparations 0.5 cc. was added to similar fermentation tube media. All the tubes were incubated for forty-eight hours at 37° C.

Out of the thirty-seven food preparations which were submitted for examination, twenty-seven were stated to have contained 0.1 per cent of sodium benzoate. In one other instance sodium benzoate was employed, but the amount added was not stated. It is believed that in most if not all the instances where sodium benzoate was employed as a preservative, sterilization by heat was also practiced, but on this point I have no actual proofs.

It is noteworthy that of the twenty-seven samples of preparations preserved with the aid of sodium benzoate in 0.1 per cent concentration, twenty-two gave indications of containing small numbers of bacteria or their spores.

The features noted in regard to the growths in the fermentation tubes were specially the degree of turbidity in the bulb and closed arm of the tube, the presence or absence of a pellicle, and the presence or absence of gas. In no instance was there any gas production in a tube. In two instances a growth was observed in saccharose bouillon; once without the formation of a pellicle, once with the formation of a pellicle. In eight instances abundant growths were observed in the fermentation tubes containing lactose bouillon, in each instance without the formation of a pellicle. In none of these eight instances of bacterial growths in the lactose bouillon fermentation tubes was there any growth in the saccharose or the dextrose tubes. In seven instances abundunt growths were observed in the dextrose bouillon fermentation tubes; three times with the formation of a pellicle, four times without the formation of a pellicle. In all seven instances mentioned the dextrose tubes alone contained growths, the saccharose and lactose tubes being free from growths. In three instances, growths were observed in all three media, with the formation of a pellicle. In two instances growths were observed

in the dextrose and the lactose fermentation tubes with the formation of a pellicle, no growth occurring in the saccharose tube. In one instance a growth, with a pellicle, occurred in the dextrose and saccharose tubes but not in the lactose tube.

These observations make it clear that the addition of sodium benzoate in 0.1 per cent concentration to the varieties of food preparations in question cannot be regarded as insuring the absence of living bacteria or their spores, notwithstanding the fact that it is probable that in most of the instances sterilization by heat had also been employed. On the other hand it is to be noted that the types of organisms which grew in the fermentation tubes were not numerous. Three different organisms which were isolated were spore-formers and it is not unlikely that all of the organisms which were obtained in the fermentation tubes belong to the spore-forming class. The spores in each instance were observed to be very resistant to the action of heat. No extended effort was made to identify the organisms observed, but the available indications (size, form, motility, behavior towards Gram-stain) make it probable that most of them belong to the subtilis group.

II. Observations were also made on the action of sodium benzoate and benzoic acid, sodium hippurate and hippuric acid on pure cultures of certain types of intestinal bacteria. The procedure employed consisted of adding these various substances to plain bouillon, to dextrose bouillon, and to dextrose bouillon containing calcium carbonate. The concentrations employed in each instance were 0.05 per cent, 0.1 per cent, and 0.2 per cent. At the end of twenty-four hours and again at the end of forty-eight hours all the tubes were examined and compared with control fermentation tubes containing media to which no preservative had been added. The results were then tabulated. The organisms employed were the following: *B. coli*, *Mic. ovalis*, *Mic. albus*, *Bact. aërogenes*, *B. infantilis* (two strains, one of which was acidophilic) and a pseudo-gas bacillus. In most instances in which benzoic acid or sodium benzoate was added the growths were somewhat inhibited but the effects observed from concentrations of 0.05 and 0.1 per cent were much less pronounced than where the concentration was 0.2 per cent. In general, inhibition was more marked from the action of sodium benzoate than in

the case of sodium hippurate[1] of the same concentration, and the inhibitory action of benzoic acid was more pronounced than that of hippuric acid. In plain bouillon slight or moderate inhibition was noticed where sodium benzoate or benzoic acid had been added in concentrations of 0.1 per cent or 0.2 per cent, but more abundant growth appeared in the tubes containing sodium hippurate and hippuric acid. In dextrose bouillon the inhibition was, in most instances, less noticeable, and in general the sodium benzoate and benzoic acid were more efficient than the sodium hippurate and hippuric acid. In those tubes to which calcium carbonate had been added to the dextrose bouillon no inhibition was noticed. Indeed the growths in these tubes were more luxuriant than was the case in control tubes and plain bouillon to which no preservative had been added. Thus the addition of calcium carbonate to the dextrose bouillon practically eliminated the inhibitory action of sodium benzoate upon the various bacteria employed. Observations were not made to determine the effect of greater concentrations of sodium benzoate. It appears likely that this action is due to the pressence of hydroxyl ions but it is possible that the presence of calcium ions exerted a definite stimulating action on the bacteria in question. Exact data are, however, lacking in regard to the operation of the calcium salt.

In the observations that were made upon the action of sodium benzoate, benzoic acid, sodium hippurate and hippuric acid on gas production by bacteria, the same procedure was followed as in the cases already mentioned, excepting that fermentation tubes were employed in place of test-tubes. Sodium hippurate and hippuric acid, in concentration of 0.1 per cent in dextrose bouillon and dextrose calcium bouillon, did not prevent the development of *B. coli* nor of *Bact. aërogenes*, although the gas formation was sometimes 25 per cent less than was observed in control tubes. Sodium benzoate and benzoic acid on the contrary, in the same concentrations, more markedly inhibited the gas formation, despite the fact that there was a distinct turbidity both in the closed arm and in the bulb of the fermenta-

[1] Hippuric acid is decomposed by many kinds of bacteria, but I think it doubtful if any considerable decomposition occurred through the action of any of the bacteria employed in these experiments.

tion tube. In dextrose calcium bouillon, on the other hand, gas production was observed despite the addition of sodium benzoate or benzoic acid; and the amount of gas formed was greater than in the case of controls grown in dextrose bouillon without the addition of calcium carbonate.

It is thus evident that the gas-forming ability of certain bacteria may be considerably reduced without a complete suppression of the power of vegetating. In general 0.05 per cent of benzoic acid exerted an effect comparable in degree to the inhibitory power noted as a result of 0.1 per cent of sodium benzoate. Similarly benzoic acid in concentration of 0.1 per cent exerted roughly speaking about the same effect as 0.2 per cent of sodium benzoate. These higher concentrations, employed in the manner just noted, not only reduce gas production but may distinctly diminish the power of reproduction of the bacteria.

In plain bouillon the action of sodium benzoate in concentration of 0.1 per cent on the growth of *B. coli* appears to be either very slight or not enough to be definitely determinable. Dr. Theobald Smith writes me that preliminary tests show that *B. coli* is not at all inhibited in bouillon by 0.1 per cent sodium benzoate and not completely checked by 0.4 per cent. Dr. Smith also states that paratyphoid bacilli and typhoid bacilli were found to be more sensitive to the benzoate than colon bacilli, but that even here the concentration of 0.1 per cent sodium benzoate does not inhibit very much.

It is clear from what has been stated that sodium benzoate in fluid media, in concentration of 0.1 per cent cannot be regarded as an effective antifermentative agent, in the sense of checking the multiplication of bacteria. This is true at least of organisms of the *B. coli* and *B. aërogenes* types and it is probably true of bacteria in general. On the other hand sodium benzoate in this concentration is not without some influence on the course of fermentation, for in addition to some restriction in multiplication there may be distinct effect, already mentioned, in diminishing the production of gas. The effect on the formation of acid, in the case of acid-forming bacteria, has not been carefully investigated but deserves attention.

The ineffectiveness of 0.1 per cent sodium benzoate in fluid or semi-fluid food preparations has been recognized at least in the

case of ketsups, where I understand it has been found necessary to use 0.2 or 0.3 per cent to protect against bacteria and yeasts.

It is possible that in semi-solid food preparations a concentration of 0.1 per cent sodium benzoate is all that is required to prevent the multiplication of ordinary microörganisms of the air. Definite knowledge of the influence of the water content of such preparations on the efficiency of the protective action of sodium benzoate is a desideratum.

III. Some observations were made on the action of sodium benzoate and benzoic acid on the mixed intestinal flora of normal individuals. The general procedure employed here was as follows: Approximately 0.1 gram of representative fecal material was suspended in sterile physiological saline solution and inoculated into fermentation tubes containing dextrose bouillon, or dextrose bouillon to which calcium carbonate had been added in excess. The concentration of the preservatives varied from 0.05 to 0.2 per cent. Control experiments were made with corresponding media containing no preservative. Observations were made upon the turbidity of the growths, on the gas production and on the character of the sediments. The tubes were incubated at 37° C. and observations were made and recorded at the end of twenty-four and forty-eight hours, respectively. The following results were obtained. Sodium benzoate in 0.1 per cent concentration and benzoic acid in 0.05 per cent concentration, in dextrose bouillon, were found to inhibit the formation of gas. Sometimes after twenty-four hours a few millimeters of gas were observed in the tubes containing sodium benzoate, and both in the case of the benzoate and the free acid moderate numbers of Gram-negative bacilli referable morphologically to the *B. coli* group were observed. A few coccal forms and some Gram-positive rods were also observed. Organisms of the bifidus type were very seldom observed. A few bacteria of acidophilic character were generally observed, but organisms of the latter type were frequently noted in fewer numbers than in the sediments of the control fermentation tubes. When the preservatives were used in higher concentrations, that is 0.2 per cent sodium benzoate and 0.1 and 0.2 per cent benzoic acid, Gram-negative forms referable to the *B. coli* type were frequently less numerous than in the control tubes. On the other hand the

coccal forms usually did not show such inhibition but, on the contrary, sometimes exhibited a marked overgrowth, especially in the case of the Gram-positive forms. Sometimes there was a slight increase in the numbers of Gram-negative bacilli and cocci, but this was an inconstant modification.

In dextrose bouillon to which calcium carbonate had been added gas formation was distinctly increased. The amount of gas was usually greater than in the control fermentation tubes containing calcium carbonate. The Gram-negative forms of the *B. coli* type and some other Gram-negative forms accumulated more abundantly in the fermentation tubes than in dextrose bouillon fermentation tubes containing preservative but no calcium carbonate.

It thus appears that sodium benzoate and benzoic acid in the concentrations employed in these experiments have the power of diminishing the gas production in fermentation tubes and at the same time modifying somewhat the character of the fermentation tube sediments. The chief modification appears to be an inhibition in the growth of organisms of the *B. coli* type and a relative increase in coccal forms of bacteria. In fermentation tubes containing calcium carbonate, the Gram-negative colon-like organisms were not inhibited as in the case of tubes containing no calcium carbonate.

In this connection may be mentioned the action of sodium benzoate on the gas-forming properties of human mixed intestinal bacteria, as reported by me in the research from my laboratory published in Bulletin No. 88 of the United States Department of Agriculture. It was observed that the fecal flora of persons taking several grams of sodium benzoate daily no longer produced as much gas as previously (when smaller quantities of sodium benzoate had been taken) in dextrose bouillon. There appeared to be no doubt as to the relation between the taking of the benzoate and the diminished gas production. The preparatory period on small doses of sodium benzoate does not seem to be necessary for the development of this effect of larger doses. One of our subjects was especially studied with reference to this point. The administration of four grams daily of sodium benzoate for several days was soon followed by a diminished power of gas production in each instance in a series of such trials.

It should also be noted that the periods of diminished gas production appeared to correspond to a change in the nature of the fermentation tube sediments, a change characterized by the appearance of an increased prominence of coccal forms of bacteria. The basis of this statement is as follows: The fermentation tube sediments were examined by me without any knowledge of the position of these preparations in the series to which they belonged. Despite this random examination of the fermentation tube sediments, it was possible to form a fairly accurate judgment in the case of each subject of the position of the slide in the series with respect to the period of low gas production. This judgment was also obtained through guidance by the relative overgrowth of coccal forms. In the slides in which such overgrowth was most marked a correspondence with a period of low gas formation was assumed and this assumption was in general justified.

It should be clearly understood that the increase in coccal forms observed in the fermentation tube sediments was not associated with any demonstrable increase of coccal forms in the fecal smears made direct from the feces.

I am unable to offer any satisfactory explanation of the depression of gas production in fermentation tubes, following the use of considerable doses of sodium benzoate. It seems probable that the phenomenon can be reproduced in monkeys, and if this is the case an explanation of this peculiar effect of the sodium benzoate might, perhaps, be obtained from a study of the bacteria and of the chemical conditions at various levels of the digestive tract. Perhaps the direct action of sodium benzoate on the intestinal bacteria in the upper part of the digestive tract is answerable for the poor gas production.

Dr. Dakin was so good as to make some observations for me on the action of sodium benzoate upon the activity of brewer's yeast in a beerwort medium. The medium originally contained 7.14 per cent of glucose (or reducing sugars calculated as glucose). After fermentation for forty-eight hours at body temperature the medium contained 1.4 per cent of glucose. A portion of the medium to which 0.15 per cent sodium benzoate had been added contained 4.33 per cent glucose, thus showing a considerable inhibiting action on the part of the benzoate. The medium fermented without benzoate contained 5.50 per cent alcohol at

the end of the experiment. The medium fermented in the presence of sodium benzoate contained 3.14 per cent alcohol. The volatile acids (calculated as acetic) were 0.01 per cent after fermentation without benzoate, and 0.02 per cent[1] after fermentation in the presence of sodium benzoate. The non-volatile acids (calculated as succinic acid) amounted to 0.05 per cent in the medium fermented without benzoate present, and to 0.02 per cent in the medium fermented in the presence of benzoate.

These figures thus indicate that sodium benzoate in concentration of 0.15 per cent inhibits markedly the fermentative activity of the yeast plant in a favorable medium, while permitting a considerable formation of alcohol. It was thought that perhaps the diminution in gas production caused by the presence of sodium benzoate might be associated with some increase in the non-volatile acids, especially lactic and succinic but this experiment gives no support for this possibility.

CONCLUSIONS.

1. Commercial food preparations to which sodium benzoate had been added in concentration of 0.1 per cent were found in most instances to contain small numbers of bacteria, chiefly of the spore-bearing kind.

2. Sodium benzoate in dextrose bouillon, in concentration of 0.1 per cent only slightly or moderately inhibits *B. coli* and other intestinal bacteria. Gas-production may, however, be considerably inhibited.

3. When mixed fecal bacteria from normal adults (on a mixed diet) are introduced into dextrose bouillon fermentation tubes containing 0.1 per cent or 0.2 per cent sodium benzoate, the bacteria are unequally inhibited. In general the organisms of the *B. coli* group appeared to be more inhibited than the coccal forms of bacteria.

I desire to acknowledge valuable aid given me by Mr. A. I. Kendall, lately Fellow of the Rockefeller Institute, in the bacteriological work on which this paper is founded.

[1] After removal of benzoic acid by chloroform extraction.

EFFECTS OF THE PRESENCE OF CARBOHYDRATES UPON THE ARTIFICIAL DIGESTION OF CASEIN.

By N. E. GOLDTHWAITE.

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The present article is one of a series undertaken at the desire and inspiration of Prof. L. E. Holt, and devoted to the problem of "Infant Feeding." The ultimate aim of the work is to determine the conditions under which cow's milk may be most conveniently utilized by infants as a foodstuff that will enable their normal growth. Many of these conditions have been established empirically. They consisted principally in modifying the natural composition of cow's milk by the addition of various substances. It was natural to begin the work by investigating the influence exercised by the milk modifiers on the process of assimilation of the infant. This process is dependent on a number of factors. The milk modifier may exercise an influence on either one or another of them. One of the first factors concerned in the process of assimilation of foodstuff is its digestion in the stomach. The rate of this digestion is influenced by modifying either the intensity of gastric secretion and pepsin production, or by the digestibility of the foodstuff. Thus every milk modifier is of service either through its physiological effect on gastric secretion or through its chemical influence on the components of the milk, rendering them more susceptible to the action of gastric juices.

Carbohydrates are the substances generally employed for modifying milk, and the present investigation is aimed to establish the influence of carbohydrates on the rate of peptic digestion of the principal component of milk, namely, casein. The influence

of carbohydrates on the rate of gastric secretion was the subject of a separate investigation.[1]

COMPARISON OF METHODS EMPLOYED FOR ESTIMATION OF PROTEOLYTIC ACTION.

Preliminary to carrying out this investigation it was deemed best to examine closely the methods commonly employed for measuring proteolytic action. For such work it was necessary to find methods which would be capable of showing unmistakably and accurately delicate differences in the rate of digestion in comparatively small periods. The differences shown should be as great as possible to avoid a relatively high percentage of error. With this object in view experiments were carried out on the peptic digestion of egg-albumen using the well known methods: (1) coagulation method of precipitating proteids by heat in the presence of sodium sulphate; (2) precipitation of proteids by tannic acid following the direction worked out by Bigelow and Cook;[2] (3) precipitation of proteids by zinc sulphate;[3] (4) the titration method;[4] (5) Salm's[5] method of measuring the hydrogen concentration by means of indicators were tested. The method involving the use of formalin before attempting to titrate[6] was published after the experiments herein recorded were completed and hence was not tested. The details of the methods employed and some of the data obtained from these experiments and also a comparison of results are given further on.

PREPARATION OF EGG-ALBUMEN SOLUTION.

A given weight of Merck's dried egg-albumen was ground up little by little, in a mortar with $\frac{N}{20}$ hydrochloric acid. The resulting thick liquid was transferred to a 500 cc. or 1000 cc. graduated flask and the volume completed with $\frac{N}{20}$ acid. The contents of the flask were thoroughly mixed and

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[1] T. Wood Clark: *Amer. Journ. of Med. Sci.*, 1909, p. 872.

[2] Bigelow and Cook: *Journ. of the Amer. Chem. Soc.*, xxix, p. 1497, 1907.

[3] Bömer: *Zeitschr. f. anal. Chem.*, 1895, xxxiv, p. 562; Zunz: *Zeitschr. f. physiol. Chem.*, xxviii, p. 219, 1899.

[4] Volhard: *Beitr. z. chem. Phys. u. Path.*, vii, p. 120, 1906. Long: *Journ. of the Amer. Chem. Soc.*, xxix, p. 225, 1907.

[5] Salm: *Zeitschr. f. physikal. Chem.*, lvii, p. 471, 1907.

[6] Sorensen: *Compt. rend. laboratoire de Carlsberg*, vii, p. 1, 1907.

then centrifugalized for ten minutes at 3000 revolutions per minute. The supernatant liquid was decanted from the sediment and filtered through dry filter paper. This filtrate was divided accurately into three equal portions. These three portions (*a*, *b* and *c*) were used for each set of experiments.

THE DIGESTION MIXTURES.

Equal amounts of pepsin (Fairchild pepsin scales dissolved in $\frac{N}{20}$ hydrochloric acid) were added to each of the three portions, *a*, *b* and *c*. A sufficient quantity of each portion was set aside for making a nitrogen determination (Kjeldahl-Gunning) and an estimation of the initial digestive value, as will be described below. The remainder of the solutions were allowed to digest at 37° C, and after 3, 6, and 24 hours, samples of each portion, after thorough shaking, were withdrawn and filtered through dry filter paper. Definite volumes of these filtrates were measured off by burettes and employed for the following experiments.

TEST METHODS.

Titration method. Ten cubic centimeters of the filtered digestion mixture were titrated with $\frac{N}{10}$ sodium hydroxide, using phenolphthalein as indicator. Phenolphthalein was the only indicator upon which any reliance could be placed. The end point is somewhat difficult to recognize, but with experience very concordant results are obtainable. The results obtained by this method are recorded in Table I.

Coagulation method. Ten cubic centimeters of the filtered digestion mixture were transferred to a 50 cc. graduated flask. To this was added 10 cc. of a saturated solution of sodium sulphate and a quantity of $\frac{N}{10}$ sodium hydroxide mixture to exactly neutralize the acidity. The required quantity was determined on a separate sample. The flask was then heated for five minutes in a boiling water-bath. After cooling the volume was made up to 50 cc. and filtered through a dry paper. 225 cc. of the clear filtrate (corresponding to 5 cc. of the original digestion mixture) were used for a nitrogen determination.

Tannic Acid method. Ten cubic centimeters of the filtered digestion mixture were transferred to a 50 cc. graduated flask. To this was added 25 cc. of a concentrated solution of sodium chloride. This mixture was cooled to 1° C. and to it was then added 15 cc. of a 24 per cent solution of tannic acid previously cooled to the same temperature. The contents of the flask were well shaken and kept at 1° C. over night, and then filtered. Twenty-five cubic centimeters of the clear filtrate were used for a nitrogen determination.

Zinc Sulphate method. Twenty-five cubic centimeters of the filtered digestion mixture were transferred to a 50 cc. graduated flask. To this was added 35 grams of zinc sulphate crystals, and the volume completed with 5 per cent sulphuric acid saturated with zinc sulphate. The flask

TABLE I

DIGESTION-SOLUTION	A			B			C			D		
I. Strength of digestion-solution per 100 cc.	2 g. egg albumen 0.4 g. pepsin			2 g. egg albumen 0.4 g. pepsin			2 g. egg albumen 0.4 g. pepsin			6.6 g. egg albumen 0.44 g. pepsin		
II. Total $\frac{N}{10}$ in 5 cc. of digestion solution measured in cc. of $\frac{N}{10}$ acid used up by ammonia distillate.	7.9			7.			8.5			23.6		
III. Titration of 10 cc. fraction of digestion-solution with $\frac{N}{20}$ NaOH....	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
1. At once.........	11.65	11.65	11.55	11.0	10.85	10.85	10.8	10.85	10.85	11.0	11.0	11.0
2. 3 hours.........	12.7	12.6	12.5	11.55	11.45	11.45	11.75	11.8	11.8	11.5	11.45	11.5
3. 6 hours.........	13.2	13.25	13.05	12.25	12.25	12.2	12.1	12.2	12.2	11.7	11.7	11.7
4. 24 hours.........	14.2	14.2	14.1	13.65	13.55	13.55	13.35	13.25	13.3	13.3	13.3	13.25
5. 72 hours.........	14.9	14.8	14.8	13.95	14.0	14.0				14.2	14.2	14.2

was well shaken, allowed to remain at room temperature over night and filtered. Ten cubic centimeters of the clear filtrate corresponding to 5 cc. of the original digestion mixture were used for the nitrogen determination.

The results obtained by these last three methods are summarized in Table II. The fact that the nitrogen determinations of any two solutions made from the same quantities of egg-albumen frequently show lack of agreement is easily accounted for when the difficulty experienced in filtering these thick solutions is considered.

A critical examination of Table IV shows a fair concordance in the three experiments comprising any given set. It shows also that the proportion of digestion taking place during a single period is small. The zinc sulphate method claims superiority over the others here considered, in that the results obtained by it show rather greater differences, whether the digestion has progressed for 3, 6, or 24 hours. These differences are all shown in Table V. The results by the zinc sulphate method are also more concordant than those obtained by any of the other methods and at the same time it is the easiest one to carry out. In the sodium sulphate method it is very difficult to establish with certainty the neutral point of the solution. In the tannic acid method, the sulphuric acid digestions (Kjeldahl) are difficult, owing to the very large amount of organic matter to be oxidized. In the zinc sulphate method, care must be taken to add sufficient fixed alkali to redissolve the zinc hydroxide precipitate first formed previous to its distillation in the Kjeldahl process; otherwise, much bumping occurs.

An attempt was made to apply the method worked out by Salm,[1] to these digestion mixtures, for measuring the hydrogen concentration by means of indicators. For this purpose, Salm's standard solutions of $\frac{N}{10}$ sodium dihydrogen phosphate were carefully prepared. The hydrogen concentration of these solutions varies from 1.10^{-1} normal to 1.10^{-10} normal. With these standard solutions the results described by Salm in his Table II were readily confirmed. However, it was impossible to obtain satisfactory results, when an attempt was made to apply these

[1] Salm: *loc. cit.*

TABLE II.

All results given in cc. of $\frac{N}{10}$ acid used up by ammonia distillate from 5 cc. of digestive-solutions:

DIGESTION-SOLUTIONS.	A			B			C			D		
I. Strength of digestion-solution per 100 cc.	2 g. egg alb. .4 g. pepsin			2 g. egg alb. .4 g. pepsin			2 g. egg alb. 4 g. pepsin			6.6 g. egg alb. .44 g. pepsin		
II. Total nitrogen. . . .	7.9			7.			8.5			23.6		
III. Methods.	Coagulation			Tannic acid			Coagulation			Coagulation		
Fractions.	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
IV. Results of digestion:												
1. At once (i.e., allowing no time for digestion). . .	4.3	4.25	3.95	1.2	1.35	1.15	3.4	3.3	3.3	6.6	6.45	6.6
2. 3 hours	6.2	5.95	6.05	1.6	1.6	1.7	4.4	4.6	4.7	7.5	7.5	7.7
3. 6 hours.	6.85		6.5	2.2	2.15	2.0	5.1	5.3	5.15	7.65	7.8	7.8
4. 24 hours.	7.9	7.9	7.75	3.15	3.05	3.1	7.2	7.2	7.3	9.6	9.6	9.7
5. 48 hours.				3.5	3.45	3.6				11.2	11.2	11.2
6. 72 hours.												

DIGESTION-SOLUTIONS.	E			F″			F′			G			G′		
I. Strength of digestion-solution per 100 cc.	6 g. egg alb. .44 g. pepsin			6 g. egg alb. .88 g. pepsin			6 g. egg alb. .88 g. pepsin.			8 g. egg alb. .279 pepsin			8 g. egg alb. .27 g. pepsin		
II. Total nitrogen. . . .	21.6			21.2			21.2			28.6			28.6		
III. Methods.	Zinc sulphate			Tannic acid			Coagulation			Coagulation			Zinc sulphate		
Fractions.	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
IV. Results of digestion:															
1. At once (i.e., allowing no time for digestion). . .	3.3	3.315	3.3	1.0	1.1	1.0	9.3	9.1	9.	8.1	8.65	8.55	3.3	3.5	3.4
2. 3 hours	4.255	4.3	4.25	1.5	1.675	1.675	9.85	9.7	9.65	9.0	9.2	9.2	3.9	3.85	3.95
3. 6 hours.	5.0	5.165	5.075	2.05	2.1	2.15	10.2	10.3	. . . . .	9.4	9.55	9.6	5.1	5.2	5.1
4. 24 hours.	8.0	8.1	8.0	4.5	4.5	4.5	14.1	14.2	14.35	12.6	12.85	12.7	8.2	8.	8.
5. 48 hours.															
6. 72 hours.	13.1	13.075	13.2	6.9	6.9	7.1	17.3	17.75	17.55	16.3	16.6	16.8	12.6	12.7	12.6

indicators to solutions containing proteins either in form of acid peptones in varying concentrations, or of partially digested egg-albumen. Under these conditions the color changes take place very gradually, and the end points of the reaction cannot be determined with certainty.

An attempt was then made to find an indicator that would show a sharp change in color in a protein solution on titration to some definite hydrogen concentration. All indicators recommended by Salm for measuring hydrogen concentration corresponding to the degree of acidity employed in our experiments, proved of little value for titration of solutions containing protein. Phenolphthalein was the only indicator which gave a fairly satisfactory end reaction.

The results which we obtained made it evident that the zinc sulphate method was the only one adapted to our purpose, and was, therefore, the only one used in our future experiments.

EFFECT OF PRESENCE OF CARBOHYDRATES UPON THE ARTIFICIAL DIGESTION OF CASEIN.

Some work had already been done by previous investigators on this subject.[1] Ogata in 1885 found that the presence of sucrose retards the digestion of meat. This was confirmed by Mugdan[2] in 1891. The results obtained by Laborde[3] in 1899 showed that dextrose retards digestion. In 1902 Nirenstein and Schiff[4] working on artificial digestions by means of Mett's tube experiments, reached a similar conclusion. These results were also confirmed by Fujitani[5] in 1905. However, Pone[6] in 1907 also working with Mett's tubes arrived at an opposite conclusion. The results recorded in the present paper were obtained by the artificial peptic digestion of casein. Solutions of casein in $\frac{N}{10}$ hydrochloric acid were used.

The method employed to determine the amount of digestion taking place in the solutions was a modification of the zinc sul-

[1] Ogata: *Arch. f. Hyg.*, vol. iii, p. 211, 1885.
[2] Mugdan: *Berliner klin. Woch.*, vol. xxviii, p. 788, 1891.
[3] Laborde: *J. Pharm.*, vi, 10, p. 484.
[4] Nirenstein and Schiff: *Arch. f. Verdauungskr.*, viii, p. 560, 1902.
[5] Fujitani: *Arch. intern. de pharm. et therap.*, xiv, p. 29, 1905.
[6] *Ibid*: xvii, p. 249, 1907.

phate method already described. Precipitation with saturative zinc sulphate could not be applied to casein solution, for the reason that the precipitate formed was of a nature that made satisfactory filtration impossible.

The casein, and casein carbohydrate digestion mixtures were precipitated with a 2.5 per cent sulphuric acid containing 50 per cent zinc sulphate before and after peptic digestion for 6, 12, and 24 hours. A nitrogen determination was made on the several filtrates. The results are recorded in cubic centimeters of $\frac{N}{10}$ acid required. That the total nitrogen contents of the various solutions remained unaltered during the experiment is also made evident in the table.

Preparation of the alkaline casein solutions. Ten grams of casein (Kahlbaum's "K") were made into a paste with 50 cc. water in a mortar and triturated with 40 cc. of $\frac{N}{5}$ sodium hydroxide. Five liters of casein solution were prepared in this manner and preserved by the addition of 10 cc. of toluol, and the whole kept at 1° C. Care was taken when withdrawing a sample to take same from near the bottom of the bottle so as to prevent any toluol from entering the pipette.

Pepsin solution. A 4 per cent solution of pepsin (Fairchild's scales) in water was prepared, a quantity of toluol equivalent to that used for preserving the casein solution was added, and the whole was kept in a refrigerator at 1° C. The digestive power of this pepsin solution did not deteriorate as was made evident by repeated Mett's tube experiments carried out under identical conditions during two weeks.

Concentrations of solutions used for digestion. Numerous trials showed that the best results were obtained with a 5 per cent solution of casein and a 0.5 per cent solution of pepsin in $\frac{N}{20}$ hydrochloric acid. To this casein pepsin hydrochloric acid mixture was added a sufficient amount of the carbohydrate to form solutions of 5, 10, and 20 per cent.

Method of preparing digestion-solution. Much difficulty was experienced in handling the digestion solution, but after repeated trials the following method was adopted: 50 grams of the stock-solution of casein were weighed in a 100 cc. graduated flask. The desired weight of carbohydrate was added to this solution. After

complete solution of the carbohydrate $\frac{N}{10}$ hydrochloric acid was added in a quantity to bring the final acidity of the solution to $\frac{N}{20}$, when the solution reached a volume of 1000 cc. (The addition of the acid to the alkaline casein solution causes a precipitation of the casein which can be redissolved upon vigorous shaking and slight warming.) The flask was allowed to cool to room temperature and 12.5 cc. of the 4 per cent pepsin solution added and sufficient water to raise the volume to 100 cc.

The digestions. It was found best to carry out each digestion in separate fractions—that is—using a separately prepared solution for each 6 hour or 24 hour digestion. In the experiments recorded in Table I, however, a total volume of 100 cc. was prepared, and at the stated intervals 25 cc. of the solution were drawn off, for precipitation by zinc sulphate. All solutions were heated to 37° C. before adding the enzyme. Toluol was added as antiseptic, the mixture carefully shaken, and the flask with contents placed in the thermostat at 37° C. for a period of 6 to 24 hours as the case might be. It was noted that within a few minutes after placing in the thermostat, the solutions acquired a gelatinous character. As the digestion proceeded this gelatinous mass gradually liquefied. This semi-solid character of the mixture interfered with the exact measuring of the solutions for digestion experiments and for this reason it was found preferable to prepare separate mixtures for each experiment. Some of the discrepancies to be noted in Table I may be accounted for by this difficulty in exactly measuring the samples drawn off.

Total nitrogen. In order to determine the total nitrogen, a digestion solution was prepared in the manner already described, then 20 cc. of this (burette measurement) was transferred to a 10 cc. flask and diluted with distilled water to 100 cc. Exactly 25 cc. (also burette measurement) of the solution (an amount equivalent to 5 cc. of the original digestion-solution) was transferred to a Kjehldahl flask, and the nitrogen determined in the usual manner.

Precipitation of proteids insoluble in a 50 per cent zinc sulphate 2.5 per cent sulphuric acid solution. Throughout this work the rate of digestion was measured by the rate of formation of protein soluble in a 50 per cent zinc sulphate solution. This was accomplished by estimating the nitrogen content of the filtrates

obtained after treating the mixtures with equal volumes of a concentrated solution of zinc sulphate. Estimations were made on samples obtained immediately after the mixtures were prepared for digestion, and on samples taken after definite periods of digestion. Repeated experiments showed the following to be the most accurate method: 25 cc. samples of the digestion-solution were prepared as already described, an equal volume of saturated zinc sulphate in 5 per cent sulphuric acid solution was added immediately, the mixure thoroughly shaken and filtered at once; then the nitrogen-content of an aliquot portion of the total volume (50 cc.) was determined by the Kjeldahl method, for which 10 cc. samples of the filtrate (corresponding to 5 cc. samples of the original digestion-solution) were employed. In experiments with digested mixtures the further digestion was arrested by heating the solution for five minutes to 70° C. The subsequent precipitation by zinc sulphate and treatment of filtrate was as above described.

The results of digestion recorded in Table IV were obtained according to the above methods. Those recorded in Table III Part A, were gotten from filtrates obtained by precipitation of 25 cc. samples taken from 100 cc. volumes of digestion-solution. the sample withdrawn not having been heated to 70° C., and the total mixture being allowed to stand over night *before* filtration. The results of Part B, Table IV, were obtained from separate 25 cc. samples, each sample being subjected to either 6, 12, or 24 hours digestion.

That the results recorded in Table IV are in closer agreement is readily discernible.

CONCLUSIONS.

A critical examination of Tables I and II shows, first, that the addition of any of the carbohydrates tested to the digestion-solution, retards the digestion; second, that this retardation is in proportion to the amount of added carbohydrate. The few apparent exceptions to these results are so completely overshadowed by the majority of retardations that the exceptions can probably be accounted for in some other way, especially in the case of Table I, in which the precipitated mass was allowed to

TABLE III.

All results given in cc. of $\frac{N}{10}$ acid used up by ammonia distillate from 5 cc. of digestion-solutions.

Digestion-solutions.	Part A. I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	Part B. XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI
I. Carbohydrate used	0																				
1. Glucose.......		5%	5%	10%	10%	20%	20%											10%	10%		
2. Maltose.......								5%	5%	10%	10%	10%	10%	20%	20%	10%	10%				
3. Dextrose																				10%	10%
II. Total Nitrogen (average) Const. for I–XXI	26.5																				
III. Results of digestion																					
1. At once (average) Const. for I-XXI .	1.6																				
2. Six hours	12.0		11.35	9.7	9.625	10.75	10.75	10.7	10.15	11.9	12.3	10.9	11.75	11.35	11.2	11.25	9.8	8.15	8.15	8.7	8.55
3. Twenty-four hours	15.8	14.85	15.35	15.275	15.275	15.62	15.7	17.55	16.85	14.475	15.7	15.05	15.55	15.2	15.2	14.0		14.0	13.9	14.75	14.6
IV. Differences																					
a. between 0 and 6 hours.	10.4		9.75	8.1	8.025	9.15	9.15	9.1	9.55	10.3	10.7	9.3	10.15	9.75	9.6	8.65	8.2	6.55	6.55	7.1	6.95
b. between 0 and 24 hours	14.2	13.25	13.75	13.675	13.675	14.0	14.1	15.05	15.25	12.875	14.1	13.45	13.95	13.6	13.6	12.4		12.4	12.3	13.15	13.0

TABLE IV.

Results in same units as in Table I.

DIGESTION-SOLUTIONS	XXII	XXIII	XXIV	XXV	XXVI	XXVII	XXVIII	XXIX	XXX	XXXI	XXXII	XXXIII	XXXIV	XXV	XXXVI	XXXVII
I. Carbohydrates acid	0															
1. Maltose		5%	10%	20%										5%	10%	20%
2. Glucose					5%	10%	20%									
3. Dextrine								5%	10%	20%						
4. Galactose											5%	10%	20%			
II. Total Nitrogen (See Table I)	26.5															
III. Results																
1. At once	1.8	1.9	1.6	1.9	1.85	1.8	1.85	1.7	1.45	1.45	1.5	1.3	1.2	1.2	1.2	1.9
2. Six hours	11.9	11.45	11.2	11.45	10.4	10.00	8.8	12.2	11.9	8.6	10.2	9.25	8.25	10.7	9.6	9.75
3. Twenty-four hours	15.85	......	......	......	15.8	14.65	13.9	......	......	......	......	......	......	14.45	13.8	13.5
IV. Differences																
a. Between 0 and 6 hours	10.1	9.55	9.6	9.55	8.55	8.2	11.05	10.5	9.45	7.15	8.7	7.95	7.05	13.25	8.4	7.85
b. Between 0 and 24 hours	14.05	......	......	......	13.95	12.85	12.05	......	......	......	......	......	......	13.25	12.6	11.6

TABLE V

Differences of results given in Table II.

DIGESTION-SOLUTIONS	A			B			C			D			E			F			F′			G			G′		
Fractions	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
0–3 hours	1.9	1.7	2.1	.4	.25	.55	1.0	1.3	1.4	.9	1.05	1.1	.955	.985	.95	.5	.575	.675	.55	.6	.65	.9	.55	.85	.6	.55	.55
0–6 hours	2.55		2.55	1.0	.8	.85	1.7	2.0	1.85	1.05	1.35	1.2	1.7	1.85	1.775	1.05	1.0	1.15	.9	1.2		1.3	.9	1.15	1.8	1.7	1.775
0–24 hours	3.6	3.65	3.8	1.95	1.7	1.95	3.8	3.9	4.0	3.0	3.25	3.1	4.7	4.785	4.7	3.5	3.4	3.5	4.8	5.1	5.35	4.5	4.2	4.15	4.9	4.5	4.7
0–48 hours				2.3	2.1	2.45				4.6	4.75	4.6															
0–72 hours													9.86	9.66	9.9	5.9	5.8	6.1	8.0	8.65	8.55	8.2	7.95	8.25	9.3	9.2	9.2

stand, as already indicated, in contact with the zinc sulphate solution over night—the 6 hour digestion solutions for about 20 hours and the 24 hour digestion solution about 24 hours. Evidently when this contact was allowed, some peptic digestion still continued even in the presence of zinc sulphate.

THE QUANTITATIVE SEPARATION OF CALCIUM AND MAGNESIUM IN THE PRESENCE OF PHOSPHATES AND SMALL AMOUNTS OF IRON DEVISED ESPECIALLY FOR THE ANALYSIS OF FOODS, URINE AND FECES.[1]

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INTRODUCTORY.

The following method was devised on account of the difficulty and inaccuracy of our present methods for determining calcium and magnesium, more especially in urine, foods and feces where these elements occur in the presence of phosphates and very small traces of iron. The method very commonly used is that of precipitation of the calcium as oxalate in dilute acetic acid and subsequent precipitation of the magnesium as magnesium ammonium phosphate. This method was described at least as long ago as 1860 in the third edition of Fresenius' text-book on quantitative analysis and used to separate these elements even in the absence of phosphates, although it was known to be somewhat inaccurate as compared with the method of double precipitation. It was stated that some magnesium came down with the calcium and some calcium stayed in solution, but that the two errors about balanced so that the results were fairly correct. The researches of Professor Richards a few years ago,[2] which

[1] Awarded the Boylston Prize of the Harvard Medical School, January 1, 1909.

[2] T. W. Richards, C. F. McCaffrey, and H. Bisbee: The Occlusion of Magnesic Oxalate by Calcic Oxalate and the Solubility of Calcic Oxalate. *Proceedings of the American Academy of Arts and Sciences*, xxxvi, p. 377, 1901.

showed that the double precipitation method as ordinarily carried out gives results which are somewhat inaccurate, suggested studying the accuracy of the acetic acid method again. It was found that the results are very inaccurate when carried out according to the descriptions given in the text-books. A method was finally devised, however, which gives very accurate results and which is at the same time fairly rapidly and easily carried out. It is more convenient and more accurate even in the absence of phosphate than the double precipitation method.

On account of the greater ease of manipulation of the smaller quantities, in the preliminary experiments amounts of solution were used which gave weights of calcium oxide and magnesium pyrophosphate of only 0.1 and 0.05 gram. For the final experiments the resulting weights were, of course, larger — about 0.22 gram. It is perhaps needless to say that the greatest precautions were taken to preserve accuracy in every detail. These minute details will, therefore, not be gone into. The experiments are described in their logical order and not exactly in the order in which the work was carried out. In attempting to reach closer and closer approximates to the best conditions some of the earlier analyses had to be repeated after varying the conditions slightly.

EXPERIMENTAL PART.

1. *The Solubility of the Oxalates and Phosphates of Calcium and Magnesium in Dilute Acetic Acid.*

(*a*) Determination of the strength of the solutions. Solutions of chemically pure calcium chloride and magnesium chloride were made up and the amounts of calcium and magnesium in 50 cc. of each determined as follows:

To the calcium solution,[1] 1 cc. of 40 per cent hydrochloric acid, 15 cc. of 2.5 per cent oxalic acid solution and a drop of alizarine were added and the solution heated to boiling on the electric stove. To the boiling solution dilute ammonia was added drop by drop until the solution was just alkaline, then 10 cc. of 3 per cent ammonium oxalate solution, and the solution set aside at the room temperature for three hours. The solution was then filtered and the precipitate washed free from chloride with cold 1

[1] Richards, McCaffrey and Bisbee: *loc. cit.*

per cent ammonium oxalate solution,[1] dried and incinerated to constant weight in a platinum crucible with the aid of the blast lamp.

Results:

No. 229	0.0958	No. 84	0.0959
" 230	0.0958	" 85	0.0958
" 231	0.0958		

To the magnesium solution, one drop of alizarine and a few drops of concentrated hydrochloric acid were added, then a slight excess of 2 per cent sodium acid phosphate solution, then 10 per cent ammonia drop by drop with constant stirring until just alkaline, and then slowly and with constant stirring enough ammonia to make the solution contain one-third its bulk of 10 per cent ammonia. The solution was allowed to stand over night at the room temperature, and the next day was filtered; washed with alcoholic ammonia (1 part alcohol, 1 part 10 per cent ammonia, 3 parts water), ignited and weighed as magnesium pyrophosphate.

Results:

No. 5	0.0564	No. 208	0.0564
" 6	0.0564	" 209	0.0564
" 7	0.0563	" 210	0.0563
" 8	0.0563		

(*b*) Separation of calcium and magnesium in the presence of phosphates in dilute acetic acid according to Fresenius. In order to see how inaccurate results were obtained by the old method, to 50 cc. of the calcium solution and 50 cc. of the magnesium solution were added 17 cc. of sodium acid phosphate—enough to combine with all the calcium and magnesium to form phosphate—and the separation carried out as follows:

A few drops of alizarine were added as indicator, then dilute ammonia until the solution was just alkaline, then dilute hydrochloric acid drop by drop until the solution was just acid and one more drop in excess. A few grams of solid sodium acetate were then added to replace the hydrochloric acid with acetic acid and to diminish the dissociation of the acetic acid. Four cubic centimeters (an excess) of 3 per cent ammonium oxalate solution were added and the solution raised to the boiling point and kept boiling for half an hour. It was then allowed to cool, 6 more cubic centimeters of 3 per cent ammonium oxalate were added and the solution was allowed to stand four hours in the cold. The calcium oxalate was then filtered off,

[1] Distilled water or hot dilute ammonium oxalate solution dissolves some calcium oxalate.

washed free from chlorides with 1 per cent cold ammonium oxalate solution, ignited and weighed as usual.[1]

Results:

No. 9	0.0979	No. 11	0.0979
" 10	0.0978		

To the filtrate from the calcium oxalate, ammonia solution was added drop by drop with constant stirring until a crystalline precipitate of magnesium ammonium phosphate was obtained and then more ammonia was very slowly added with constant stirring until the solution contained one-fourth its bulk of 10 per cent ammonia. The solution was then allowed to stand over night in a cool place and the next day filtered, washed and ignited.

Results:

No. 12	0.0552	No. 14	0.0555
" 13	0.0553		

The calcium is evidently high and the magnesium low.

The calcium oxide obtained in analyses Nos. 9 and 10 was in each case dissolved in dilute hydrochloric acid, the solution made up to 50 cc. and the calcium reprecipitated as oxalate and weighed as before.

Results:

No. 15	0.0958	No. 16	0.0957

Precipitate No. 11 was dissolved in nitric acid and tested with ammonium molybdate for phosphate. A good precipitate of ammonium phospho-molybdate was obtained.

The filtrates from the calcium oxalate in analyses No. 15 and No. 16 were evaporated to small bulk and boiled with nitric acid to destroy the oxalic acid and the phosphate determined by double precipitation with ammonium molybdate and magnesia mixture.

Results (Weights of the magnesium phosphate obtained):

No. 18	0.0029	No. 19	0.0028

This amount of phosphate (about 0.0018 gram of P_2O_5) just about accounts for the high results in analyses Nos. 9, 10 and 11.

[1] In fact the standing in the cold and washing with ammonium oxalate solution are improvements on the details as described in the literature.

Precipitates No. 15 and No. 16 were dissolved and the calcium again precipitated to see how much the difference between Nos. 15 and 16, and Nos. 9 and 10 was to be accounted for by simple manipulation.

Results:

No. 20	0.0955	No. 21	0.0957

The loss is slight. A test of the filtrate for phosphates gave negative results.

The low magnesium results in analyses Nos. 12, 13 and 14 are probably due, as later analyses show, to the presence of salts rather than to occlusion with calcium oxalate.

The precipitation of the calcium phosphate is due to the presence of the HPO_4'' ion in the nearly neutral solution.

$$Ca^{++} + HPO_4^{--} = CaHPO_4$$

The concentration of the HPO_4'' ion can be decreased to practically nothing by adding acid,

$$H^+ + HPO_4^{--} = H_2PO_4^-$$

only, unfortunately the presence of acid makes the calcium oxalate more soluble. Obviously, then, the next thing to do was to determine if the conditions could not be so adjusted that while all the calcium would be precipitated as oxalate none would come down as phosphate.

(*c*) ADJUSTMENT OF THE CONDITIONS FOR THE COMPLETE PRECIPITATION OF PURE CALCIUM OXALATE IN THE PRESENCE OF PHOSPHATE. First, some preliminary tests were made to see how much hydrochloric acid and oxalic acid are practically neutralized by sodium acetate using alizarine as indicator, and to see how nearly neutral the solution should be to just keep calcium or magnesium phosphate in solution in the cold and also when boiled.

It was found that 10 drops of concentrated hydrochloric acid became practically neutral with 10 cc. of 20 per cent sodium acetate solution.

To see if calcium phosphate would precipitate in such a mixture, solutions of calcium chloride and sodium acid phosphate

were mixed and made just neutral to alizarine. Then 10 drops of concentrated hydrochloric acid and 10 cc. of 20 per cent sodium acetate were added. There was no precipitation in the cold during the first hour, but after two hours a small crystalline precipitate was obtained which was tested and found to be calcium phosphate. The experiment was repeated except that the solution was heated at once, when a flocculent precipitate was obtained at about 60° C. A similar test was then made but without adding sodium acetate. No precipitate was obtained even on boiling. Twenty per cent sodium acetate solution was then added drop by drop. A permanent precipitate was obtained when less than 3 cc. had been added.

To see what proportions of hydrochloric acid and sodium acetate will just hold calcium phosphate in solution, the following tests were made:

In each case 4 cc. of 10 per cent calcium chloride solution, 2 cc. of 10 per cent sodium acid phosphate solution, and 16 cc. of water were made just acid to alizarine and then ten drops of concentrated hydrochloric acid added in excess. Varying amounts of 20 per cent sodium acetate solution were then added as follows:

To No.	27,	3	cc.	of	20 per	cent	sodium acetate	solution
"	28,	4	"	"	"	"	"	"
"	29,	5	"	"	"	"	"	"
"	30,	6	"	"	"	"	"	"
"	31,	7	"	"	"	"	"	"
"	32,	8	"	"	"	"	"	"
"	33,	9	"	"	"	"	"	"
"	34,	10	"	"	"	"	"	"

In no case did a precipitate come down on standing over night at the room temperature. Each mixture was then boiled and a flocculent precipitate obtained in Nos. 29, 30, 31, 32, 33 and 34. Nos. 27 and 28 remained perfectly clear. On using 20 per cent ammonium acetate instead of sodium acetate the same results were obtained.

In order to get better adjustment of acidity, another series of tests was made using $\frac{N}{2}$ hydrochloric acid instead of concentrated hydrochloric acid.

Two cubic centimeters of 10 per cent calcium chloride solution and 2 cc. of 10 per cent sodium acid phosphate solution were mixed and made just acid to alizarine and 10 cc. of $\frac{N}{2}$ hydrochloric acid added in excess. Then

varying amounts of 20 per cent sodium acetate solution were added as follows:

To No.	43	added	3	cc.	of	20 per	cent	sodium	acetate	solution
"	44	"	3.5	"	"	"	"	"	"	"
"	45	"	4.	"	"	"	"	"	"	"
"	46	"	4.5	"	"	"	"	"	"	"
"	47	"	5	"	"	"	"	"	"	"
"	48	"	5.5	"	"	"	"	"	"	"
"	49	"	6	"	"	"	"	"	"	"
"	50	"	6.5	"	"	"	"	"	"	"

No precipitation was observed in any case after 24 hours at the room temperature. On boiling, Nos. 43, 44, 45 and 46 remained clear; Nos. 47, 48, 49 and 50 gave a precipitate.

A set of experiments similar to No. 43 to No. 46 was performed using magnesium chloride instead of calcium chloride, but in no case was a precipitate obtained even on boiling.

A few tests were then made to see how much 20 per cent sodium acetate was required to neutralize $\frac{N}{2}$ hydrochloric acid and 2.5 per cent oxalic acid. It was found that it took 12 cc. of 20 per cent sodium acetate solution to make practically neutral to alizarine 10 cc. of $\frac{N}{2}$ hydrochloric acid (the amount to be used in the analyses) in 50 cc. of water. It took 9 cc. of sodium acetate solution to neutralize 10 cc. of 2.5 per cent oxalic acid (the amount to be used in the analyses) in 50 cc. of water. Ten cubic centimeters of $\frac{N}{2}$ hydrochloric acid mixed with 10 cc. of oxalic acid solution in 50 cc. of water were neutralized by 22 cc. of 20 per cent sodium acetate solution.

To see how the addition of ammonium oxalate would affect the results, 20 cc. of 3 per cent ammonium oxalate solution were added in each case to the neutral mixtures described in the preceding paragraph. There was no perceptible change in the shade of the alizarine, so that addition of ammonium oxalate probably does not affect much the precipitation of phosphate.

The preceding tests gave some idea of about the amounts of acid and sodium acetate to use in the precipitation of the calcium oxalate and also suggested that the solution be kept cold after the addition of the sodium acetate.

The following quantitative experiments were then carried out:

To 50 cc. of the standard calcium chloride solution were added 10 cc. of $\frac{N}{2}$ hydrochloric acid and 10 cc. of 2.5 per cent oxalic acid. This is about equivalent to the amount of calcium present. The solution was then boiled for about fifteen minutes. A large part of the calcium oxalate was precipitated, and, on account of being fairly soluble in the acid solution, came down in a coarsely crystalline condition which made subsequent filtration easy. The solution was then cooled and 10 cc. of 3 per cent ammonium oxalate solution added in order to have an excess of oxalate present. Then different amounts of 20 per cent sodium acetate solution were added as follows:

To. No.	94	and No.	95	added	no sodium acetate
"	96	"	97	"	1 cc. of 20 per cent sodium acetate solution.
"	98	"	99	"	2 " " " " " "
"	138	"	139	"	4 " " " " " "
"	140	"	141	"	6 " " " " " "
"	72	"	73	"	8 " " " " " "
"	74	"	75	"	9 " " " " " "
"	76	"	77	"	10 " " " " " "
"	78	"	79	"	11 " " " " " "

Results:

No. 94	0.0945	No. 95	0.0940
" 96	0.0945	" 97	0.0946
" 98	0.0958	" 99	0.0951
" 138	0.0953	" 139	0.0953
" 140	0.0957	" 141	0.0957
" 72	0.0958	" 73	0.0957
" 74	0.0958	" 75	0.0958
" 76	0.0960	" 77	0.0958
" 78	0.0958	" 79	0.0958

It is plain that 6 cc. of sodium acetate under these conditions suffice to completely precipitate the calcium oxalate.

The next thing to determine was whether any phosphate comes down under these circumstances. Analyses Nos. 235, 236 and 237 were carried out just as Nos. 76 and 77 (with 10 cc. of 20 per cent sodium acetate solution) only, in addition, 10 cc. of 2 per cent sodium acid phosphate was added to the calcium solution.

Results:

No. 235	0.0958	No. 237	0.0959
" 236	0.0958		

It is plain that between the limits of 6 cc. of sodium acetate solution and 10 cc.—limits which are wide enough—no calcium

phosphate is precipitated. Calcium oxalate is quantitatively precipitated under these conditions.

The next thing to do was to determine the effect of the presence of magnesium on the result, but it was found necessary to study the details of determining magnesium alone first, as poor results were obtained in precipitating the magnesium directly in the filtrate from the calcium oxalate.

II. *The Precipitation of Magnesium alone as Magnesium Ammonium Phosphate.*

(*a*) THE EFFECT OF SALTS. Fifty cubic centimeters of the standard calcium solution was mixed with 50 cc. of the standard magnesium solution and the calcium precipitated as in the preceding section using 8 cc. of 20 per cent sodium acetate solution.

Results:

No. 88..............	0.0957	No. 89..............	0.0959

The magnesium was determined in the filtrate as in the case of the standard solutions.

Results:

No 90..............	0.0518	No. 91..............	0.0531

It is evident that the low results in the case of magnesium are not due to occlusion with the calcium, since the calcium determinations were correct, but in all probability to solubility, either on account of too large volume or on account of the presence of various salts.

To see how the quantities of salts used in connection with the calcium determination affect the results, eight analyses were carried out as follows:

To 50 cc. of the standard magnesium solution were added 7 cc. of concentrated hydrochloric acid, 10 cc. of 2.5 per cent oxalic acid solution, 10 cc. of 3 per cent ammonium oxalate solution, 4 cc. of 20 per cent sodium acetate solution, and 10 cc. of 2 per cent sodium acid phosphate solution. The solutions were made alkaline and the magnesium determined in the same manner as before.

Results:

No. 108	0.0539	No. 112	0.0530
" 109	0.0541	" 113	0.0524
" 110	0.0548	" 134	0.0514
" 111	0.0526	" 135	0.0516

Three analyses were then carried out similar to the preceding but in which the oxalates and ammonium salts were destroyed as follows.

Twenty cubic centimeters of concentrated nitric acid were added to each and the solutions evaporated almost to dryness. Then a few cubic-centimeters of hydrochloric acid were added and the solution heated to replace nitrates with chlorides. Most of the acid was then evaporated off, the solution diluted with water, and the magnesium determined as usual.[1]

Results:

No. 136	0.0564	No. 143	0.0564
" 137	0.0562		

It is evident that it is the presence of salts that brings about the low results in analyses Nos. 108 to 113.

(*b*) The addition of phosphate before and after the solution is made alkaline. It was found in accord with the results of Neubauer[2] that slow addition of the phosphate after the solution is alkaline gives a precipitate containing too little phosphate. The analyses that follow were carried out just as analyses Nos. 5 to 8, except that the solution was first made alkaline and then phosphate added drop by drop with constant stirring until an excess was present.

Results:

No. 100	0.0561	No. 202	0.0556
" 101	0.0561	" 203	0.0554
" 102	0.0560	" 204	0.0555
" 103	0.0560		

(*c*) The bulk of the solution; solubility of magnesium ammonium phosphate. It was found in these and later experiments that magnesium ammonium phosphate is very insoluble in

[1] In these and following experiments a small amount of ammonium chloride was always present to prevent precipitation of magnesium hydroxide.

[2] H. Neubauer: Ueber die Bestimmung des Magnesiumoxyds als Magnesiumpyrophosphate, *Zeitschrift für angewandte Chemie*, 1896, p. 435.

the strength of ammonia used. Analyses Nos. 128 to 133 bring out this point. In Nos. 128, 129 and 130 the magnesium was precipitated in the usual manner.

Results:

No. 128.............	0.0566	No. 130.............	0.0565
" 129.............	0.0565		

Each of these precipitates was then dissolved in dilute hydrochloric acid and reprecipitated in a volume of about 70 cc.

Results:

No. 131.............	0.0564	No. 133.............	0.0565
" 132.............	0.0564		

The differences are insignificant.

(*d*) Double precipitation of the magnesium. Analyses Nos. 128 to 133 show also that a double precipitation of the magnesium is not necessary when the first precipitation is carried out in a solution free from excess of salts. Nos. 144, 145, 211, 212 and 213 show the same thing. In these, the magnesium was precipitated as usual in a solution free from excess of salts and then the precipitate dissolved without igniting and reprecipitated.

Results:

No. 144.............	0.0565	No. 212.............	0.0563
" 145.............	0.0565	" 213.............	0.0563
" 211.............	0.0564		

(*e*) Rapidity of formation of the precipitate. It does not seem to make any difference in the result whether the excess of ammonia is added quickly or slowly after the solution has been made alkaline, but, when added slowly, a crystalline precipitate easy to wash and to filter is obtained. In analyses Nos. 205, 206 and 207, the excess of ammonia was added at once after the solution was made alkaline.

Results:

No. 205.............	0.0562	No. 207.............	0.0564
" 206.............	0.0564		

III. *The Separation of Calcium and Magnesium in the Presence and Absence of Phosphates.*

(*a*) IN THE ABSENCE OF PHOSPHATES. It has been seen that, using the data described in I (*c*), calcium, in the presence of phosphates, can be quantitatively precipitated as oxalate without contamination with phosphate. The next analyses were carried out to determine if the same details would permit of the quantitative separation of calcium and magnesium. Fifty cubic centimeters of the standard calcium solution and 50 cc. of the standard magnesium solution were mixed together and the calcium precipitated as in analyses Nos. 72 and 73 (10 cc. of $\frac{N}{2}$ hydrochloric acid and 8 cc. of 20 per cent sodium acetate solution).

Results:

No. 238	0.0959	No. 240	0.0959
" 239	0.0958		

The magnesium was determined in the filtrate after destruction of the salts as in analyses Nos. 136, 137 and 143.

Results:

No. 241	0.0564	No. 243	0.0565
" 242	0.0564		

It is not safe to use much less than 10 cc. of $\frac{N}{2}$ hydrochloric acid in the precipitation of the calcium, for, otherwise, magnesium is liable to be occluded. Two analyses were carried out similar to the preceding but in which 5 cc. of $\frac{N}{2}$ hydrochloric acid, 10 cc. of 2.5 per cent oxalic acid and 6 cc. of 20 per cent sodium acetate solution were used.

Results (Calcium determination):

No. 146	0.0965	No. 147	0.0965

(*b*) IN THE PRESENCE OF PHOSPHATE. Analyses were then carried out similar to those described in the preceding section except that enough sodium acid phosphate was added to combine with the calcium and magnesium.

Results (Calcium determination):		*Results (Magnesium determination):*	
No. 244	0.0958	No. 247	0.0564
" 245	0.0958	" 248	0.0564
" 246	0.0957	" 249	0.0564

IV. The Effect of Small Quantities of Iron.

As only small quantities of iron occur in urine, feces, and most food stuffs, the effect of only small quantities of iron was studied.

(*a*) Calcium determination in the presence of phosphate, magnesium and iron. Analyses similar to those described under III (*b*), except that 2 cc. of 1 per cent ferric chloride solution were added, were then carried out.

Results:

No. 170	.0958	No. 171	0.0959

(*b*) Magnesium determination in the presence of iron. Magnesium determinations were made in the standard solution to whch 2 cc. of 1 per cent ferric chloride were added.

Results:

No. 214	0.0626	No. 216	0.0629
" 215	0.0623		

These precipitates were dissolved and reprecipitated but the iron again came down.

Results:

No. 217	0.0620	No. 219	0.0625
" 218	0.0624		

(*c*) Effect of citric acid on the iron and magnesium. Remembering the effect of organic acids like citric acid on the solubility of iron in ammoniacal solutions, analyses similar to 214, 215 and 216 were repeated but in each case some sodium citrate was added. To No. 220, 0.5 cc. of 5 per cent sodium citrate was added. To Nos. 221 and 222, 1.0 cc. of 5 per cent sodium citrate solution to each.

Results:

No. 220	0.0573	No. 222	0.0571
" 221	0.0574		

As all but a small quantity of iron remained in solution, these precipitates were disolved in dilute hydrochloric acid and reprecipitated after again adding 0.5 to 1.0 cc. of 5 per cent sodium citrate solution.

Results:

No. 223	0.0564	No. 225	0.0564
" 224	0.0564		

To see what effect the citric acid has on the magnesium determination alone, determinations of magnesium were made in solutions to which in each case 1.0 cc. of 5 per cent citric acid was added.

Results:

No. 226	0.0565	No. 228	0.0564
" 227	0.0564		

It is plain that addition of small amounts of citric acid enables us to determine magnesium in the presence of small amounts of iron when double precipitation is carried out. (The amount of iron added in these analyses is more than usually occurs in the analyses of feces, urine and food.)

(*d*) Determination of calcium and magnesium in the presence of phosphate and iron. Analyses were then carried out similar to those described under III (*b*) except that in each case 2 cc. of 1 per cent ferric chloride were added, and the magnesium determined by double precipitation after addition of sodium citrate solution as in analyses 226, 227, 228.

Results (Calcium determination):		*Results (Magnesium determination):*	
No. 250	0.0958	No. 253	0.0563
" 251	0.0958	" 254	0.0563
" 252	0.0959	" 225	0.0564

V. *Final Experiments.*

In order to confirm the accuracy of the method of determining calcium and magnesium in the presence of phosphates and iron, stronger solutions of calcium and magnesium were made up and analyzed using the details found best in the preceding experiments.

(*a*) Analysis of the standard solutions. Three determinations of the calcium alone in the new solution gave:

Results:

No. 181	0.2208	No. 183	0.2208
" 182	0.2208		

Three determinations of the magnesium gave

Results:

No. 232	0.2228	No. 234	0.2231
" 233	0.2230		

(*b*) CALCIUM ALONE BY THE NEW METHOD. Determinations of calcium alone by the new method gave the following results:

Results:

No. 256	0.2209	No. 258	0.2208
" 257	0.2208		

(*c*) CALCIUM IN THE PRESENCE OF PHOSPHATES. Determination of calcium by the new method in the presence of enough phosphate to combine with it gave:

Results:

No. 259	0.2208	No. 261	0.2210
" 260	0.2208		

(*d*) CALCIUM AND MAGNESIUM IN THE PRESENCE OF EACH OTHER. Fifty cubic centimeters of the standard calcium solution were mixed with 50 cc. of the standard magnesium solution and the calcium determined by the new methods.

Results:

No. 196	0.2209	No. 198	0.2208
" 197	0.2210		

The magnesium was determined in the filtrate from Nos. 196, 197 and 198.

Results:

No. 199	0.2228	No. 201	0.2227
" 200	0.2230		

(*e*) CALCIUM AND MAGNESIUM IN THE PRESENCE OF PHOSPHATES. Analyses were then carried out similar to the preceding but enough phosphate was added to combine with all the calcium and magnesium.

Results (*Calcium determination*):		*Results* (*Magnesium determinations*):	
No. 262	0.2208	No. 265	0.2225
" 263	0.2210	" 266	0.2228
" 264	0.2209	" 267	0.2225

(*f*) Calcium and magnesium in the presence of phosphate and iron. Determinations similar to those described under IV (*d*) were repeated on the stronger solutions.

Results (*Calcium determination*):		*Results* (*Magnesium determinations*):	
No. 268	0.2209	No. 271	0.2225
" 269	0.2211	" 272	0.2224
" 271	0.2209	" 273	0.2228

SUMMARY.

A summary of the details of the method follows.

Solutions needed:

3 per cent ammonium oxalate solution.
2.5 per cent oxalic acid solution.
$\frac{N}{2}$ hydrochloric acid.[1]
2 per cent sodium acid phosphate solution.
20 per cent sodium acetate solution.[2]
Dilute aqueous ammonia solution (specific gravity 0.96).
Concentrated hydrochloric acid.
Concentrated nitric acid.
5 per cent sodium citrate solution.
Dilute alizarine solution as indicator.

To the solution containing calcium, magnesium, phosphate and iron,[3] 2 drops of dilute alizarine solution are added and then ammonia drop by drop until the solution is just alkaline. Dilute hydrochloric acid is then added drop by drop until the solution is just acid. If the solution has become warmed by the neutralization, a few drops acid in excess should be added to insure complete solution of the calcium phosphate, the solution cooled, and the neutralization repeated.

[1] This solution need not be absolutely accurately half normal.

[2] Twenty grams of the crystalline sodium acetate containing water of crystallization dissolved and made up to 100 cc.

[3] In the case of food or feces the material is first incinerated and then dissolved in dilute hydrochloric acid.

When the solution is cold, and acid to alizarine, all the calcium phosphate is in solution. A small amount of phosphate of iron may remain undissolved at this point but will go into solution when the half normal acid is next added.

After the solution is just acid to alizarine, 10 cc. of $\frac{N}{2}$ hydrochloric acid and 10 cc. of 2.5 per cent oxalic acid are added and the solution is brought to the boiling point and kept just gently boiling until the calcium oxalate is coarsely granular. The flask should be kept covered with a watch glass to prevent spattering.[1] Three per cent ammonium oxalate is then added a few drops at a time to the boiling solution and waiting after each addition until the resulting precipitate has become coarsely crystalline. The amount of ammonium oxalate to be added depends on the amount of calcium in solution. Twice the amount necessary to combine with all the calcium is sufficient.

After the calcium oxalate has become ,coarsely crystalline, and has settled to the bottom of the flask, it should be frequently stirred up in the liquid to prevent sudden boiling over. Recalling Professor Richards' hypothesis that occlusion consists in a distribution of an undissociated substance between the liquid and the nascent solid phase and his experiments in seeming accord with this hypothesis,[2] it will be seen that the conditions here are chosen so as to give the least possibility of occlusion of either magnesium oxalate or calcium phosphate, viz: the precipitation in a solution containing a relatively high concentration of hydrogen ions, the very slow addition of the ammonium oxalate, which is not present in excess until practically all the calcium is precipitated, and, later, the presence of considerable quantities of another partly dissociated salt, sodium acetate. The slow precipitation of the calcium oxalate in the boiling acid solution, in which it is partly soluble gives the best conditions for the formation of large crystals, and as a result, a very coarsely granular precipitate is obtained that is very easily filtered and washed without any danger of particles passing through the filter. The sodium acetate solution should be added very slowly and with constant stirring so that there will be no danger of a local precipitation of calcium phosphate or magnesium oxalate.

The solution is then allowed to stand in a cool place from four to eighteen hours, filtered cold, and washed free from chlorides with cold 1 per cent ammonium oxalate solution.

[1] The flasks somewhat similar to Erlenmeyer flasks but with slightly flaring top and lip are convenient for this work.

[2] See Richards, McCaffrey and Bisbee: *loc cit.*

Washing with distilled water caused a certain amount of calcium oxalate to go into solutions as did even washing with hot half per cent ammonium oxalate solution. Washing with cold 1 per cent ammonium oxalate solution did not dissolve any calcium oxalate.

The precipitate is allowed to dry and then incinerated with the filter paper in a platinum crucible. It is finally heated in the blast lamp to constant weight.

The filtrate containing the magnesium is evaporated almost to dryness after addition of 20 cc. of concentrated nitric acid. When almost dry, and fumes of nitric oxide no longer come off, 10 cc. of concentrated hydrochloric acid are added and the solution again evaporated nearly to dryness. The solution is then diluted to about 80 cc., nearly neutralized with ammonia and then cooled.

If no iron is present, enough sodium acid phosphate is added to precipitate the magnesium, if enough is not already present, and a slight amount added in excess. Ammonia is then added drop by drop with constant stirring until the solution is alkaline, and then enough more added slowly and with constant stirring to make the solution contain one-fourth its bulk of dilute ammonia (specific gravity 0.96). The solution is allowed to stand over night. Next day it is filtered and washed free from chlorides with alcoholic ammonia solution (1 part alcohol, 1 part dilute ammonia, 3 parts water). The precipitate with filter paper is incinerated slowly and carefully with good supply of air to prevent reduction in the usual manner.

If iron is present, 0.5 to 1.0 cc. of 5 per cent sodium citrate solution is added before the magnesium is precipitated. The magnesium is then precipitated and filtered as in the absence of iron. The precipitate need not be thoroughly washed but is washed a few times by decantation and then the precipitate on the filter paper and in the flask is dissolved in dilute hydrochloric acid, made up to 80 cc. with distilled water, and reprecipitated as before, after addition of 0.5 to 1.0 cc. of 5 per cent sodium citrate solution. The precipitate is filtered, washed, ignited and weighed as in the absence of iron. The directions should be very carefully adhered to in order to get the best results.

A NOTE ON THE ESTIMATION OF TOTAL SULPHUR IN URINE.

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(Received for publication, November 30, 1909.)

The subject of total sulphur estimation in urine has recently received considerable attention.[1] In view of the unsettled condition of the subject at the present time a brief discussion of the theoretical aspects of the latest contribution to the subject may not be out of place.

In a recent number of the *Biochemical Journal* Ritson[2] gives an interesting series of figures for urinary total sulphur obtained by various processes, and proposes a new method for total sulphur estimation in urine. Throughout his study Ritson fails to recognize that the greatest error in sulphate estimations arises from the tendency to obtain too high results, rather than too low. Like certain previous investigators,[3] Ritson assumes that the higher the values obtained through the use of any method, the more accurate the method. Such a standard of judgment cannot but lead to error, especially in sulphate work. An impure (and therefore too heavy) barium sulphate precipitate is obtained under a great variety of conditions, and it is to be deplored that Ritson should propose a sulphur method, the only experimental

[1] For the recent discussion, see Folin: This *Journal* i, p. 131, 1906. Schulz: *Archiv für die gesamte Physiologie*, cxxi, p. 141, 1908. Konshegg: *ibid.*, cxiii, p. 274, 1908. Osterberg and Wolf: *Biochemische Zeitschrift*, ix, p. 307, 1908. Abderhalden and Funk: *Zeitschrift für physiologische Chemie*, lviii, p. 331, 1908. Gill and Grindley: *Journal of the American Chemical Society*, xxxi, p. 52, 1909. Folin: *ibid.*, xxxi, p. 284, 1909. Benedict: This *Journal*, vi, p. 363, August, 1909.

[2] Ritson: *Biochemical Journal*, iv, pp. 337ff. September, 1909.

[3] Gill and Grindley: *Loc. cit.*

justification offered for which is that it gives higher figures than any other method will yield. Differences in urinary sulphur by *total* sulphur methods affect of course, only the figures for the neutral sulphur, and a 10 per cent difference by two methods often means that the one giving the lower results has missed from 40 to 60 per cent of the neutral sulphur, a point demanding most complete experimental demonstration when the criticism is directed against such a theoretically correct procedure as fusion with an excess of sodium peroxide. Ritson's method theoretically contains several possible sources of error, and no evidence has been presented that these may not be essential factors in the production of the high figures which he obtained. The addition of barium peroxide, or of any barium compound, to a sulphate mixture before addition of hydrochloric acid is very apt to yield precipitates which cannot be washed free from impurities,[1] and is a procedure employed in no standard method for sulphate determination. Folin has also called attention to the fact that sudden ignition of urinary residues with sodium peroxide (the procedure recommended by Ritson) is apt to give rise to the formation of nitrates, a seriously interfering substance in sulphate precipitation. Ritson's precipitation takes place in the presence of an appreciable quantity of ferric oxide, a compound (which like nitrates of the alkali metals) shows a marked tendency to contaminate barium sulphate precipitates.[2]

Ritson's comparative figures for the different methods are of much interest, but the conclusions drawn from these figures cannot be accepted without corroboration.

[1] Folin (this *Journal*, i, p. 131, 1906) has called particular attention to this fact in his discussion of Baumann's method for the determination of inorganic sulphate in urine.

[2] See Fresenius: *System of Quantitative Analysis*, under "Properties of Barium Sulphate," for corroboration of these statements. Jannasch and Richards (*Journal für Praktische Chemie* (2), xxxix, p. 321) state that no correct determination of sulphate can be made in the presence of iron.

THE FATE OF SODIUM BENZOATE IN THE HUMAN ORGANISM.

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(Received for publication, November 29, 1909.)

In spite of a large number of investigations, one encounters in the current literature the most diverse statements concerning the fate of salts of benzoic acid in the human organism. Only one fact is apparently accepted by all as settled beyond question, namely, that part of the benzoate is converted into a hippurate through union with glycocoll. Upon the question as to how nearly this reaction proceeds towards completion there appears to be no agreement of opinion; some authors contending that the conversion is practically quantitative while Brugsch and others state that in carnivorous animals and in man the amount of uncombined benzoate excreted exceeds the amount of benzoic acid paired with glycocoll.

About a year ago a careful study appeared by Lewinski[1] "Ueber die Grenzen der Hippursäurebildung beim Menschen," in which he claimed to have recovered in the form of hippuric acid practically the whole of large doses of sodium benzoate (12 to 20 grams) given to men. When extremely large doses were given (40 grams and over) a certain proportion of the benzoate was excreted unchanged in the urine which also contained reducing substances and was dextro-rotatory. At the same time Seo showed that bacterial decomposition may affect the reconversion of hippuric acid into benzoic acid and that therefore bacterial contamination of the urine was a source of error to be guarded against in investigations of the kind in question.

This work of Lewinski has been subjected by Brugsch[2] to an extremely hostile criticism. By methods which, to the writer

[1] *Arch. f. exper. Path. u. Pharm.*, lviii, p. 397, 1908.
[2] *Zeit. f. exper. Path. u. Therapie*, v, p. 731, 1909.

at least, appear of questionable character, Brugsch and Tsuchiya[1] come to the conclusion that when benzoic acid in doses varying from 4 to 24.6 grams is administered to man, only from 22 to 47 per cent of the benzoic acid is recoverable in the form of hippuric acid. It is, however, not the object of the writer to enter into any detailed discussion of Brugsch's methods or deductions but rather to record certain experimental results which entirely corroborate and in some direction amplify those of Lewinski.

The results to be recorded were obtained from experiments upon healthy young men who received their doses of sodium benzoate in the form of a 5 per cent solution, three times daily after meals. Water was permitted to be freely taken after swallowing the benzoate. The diet was carefully regulated so as to avoid the consumption of any article known to have any special influence upon the hippuric acid output.

In each case the normal hippuric acid output was estimated for at least three days previous to the consumption of the benzoate.

The urines were preserved at low temperatures by the addition of a little chloroform. A somewhat improved process for the determination of the hippuric acid was employed and the method of analysis will therefore be described in detail.

The results are comprised in the following table:

[1] *Zeit. f. exper. Path. u. Therapie*, v, p. 737, 1909.

Day		Sodium benzoate administered cal'd as benzoic acid.	Hippuric acid excreted per 24 hours cal'd as benzoic acid.	Benzoic acid excreted as hippuric acid, due to benzoate administered.	Free benzoic acid, excreted as such.	Aromatic oxy-acids per 24 hours.	Glycuronic acid.
		grams.					
Exp. I...	1		0.84		none	0.212	minimal trace
	2		0.91		"		
	3		0.83		"		
	4	10.0	8.86	19.20	"	0.202	very light increase
	5	10.0	10.89		"		
	6		1.91		"		
	7		1.02		"		
	8		0.90		"	0.217	minimal trace
Exp. II..	1, 2, 3		0.58		none	trace	trace
	4	5.0	5.27	14.8	"	no increase	no increase
	5	5.0	5.44				
	6	5.0	5.40				
	7		1.01			trace	
Exp. III.	1, 2, 3		0.62		none	trace	trace
	4	5.0	5.91	15.68	"	no increase	no increase
	5	5.0	6.08				
	6	5.0	4.79				
	7		1.48				

It will be seen that in the first experiment out of 20.0 grams of benzoic acid consumed in the form of sodium benzoate no less than 19.20 grams were recovered as hippuric acid. In each of the two other experiments 15 grams of benzoic acid was ingested in three days and here too the conversion into hippuric acid was practically quantitative. Examinations were made for free benzoic acid with negative results in every case. It is sometimes stated that a certain proportion of benzoic acid undergoes complete combustion in the animal body, although the evidence for this statement is not very clear. If this actually were the case it might be inferred that aromatic oxy-acids would represent intermediate stages in the reaction. It was of interest therefore to try and detect any such increase in the excretion

of aromatic oxy-acids. In the first experiment an attempt was made to estimate them quantitatively by a method essentially based upon their separation and subsequent titration with iodine solution. In the other experiments the intensity of the reaction with Millon's reagent of the purified residue from the extraction of the acidified urines with ether, was noted. In no case could any increase in aromatic oxy-acids be observed.

A slight increase in the normal glycuronic acid content of the urine was observed in the first experiment during the benzoate period, but the increase was slight. The urine reduced Fehling's solution feebly but on decolorization with charcoal the urine showed no visible rotation in a 200 mm. tube. The reaction with orcin and hydrochloric acid was faintly positive.

The hippuric acid estimations were made as follows: From 300 to 500 cc. of urine, depending upon the hippuric acid content, is evaporated upon the water-bath to about 100 cc. The concentrated urine is then transferred to a constant extraction apparatus, strongly acidified with phosphoric acid and then extracted with ethyl acetate for the whole of a working day. The ethyl acetate extract, which should measure at least 200 cc., contains many other substances besides hippuric acid. The extract is transferred to a separating funnel and thoroughly shaken with four successive portions of saturated salt solution, using in all 75 cc. By this means a considerable amount of urea is removed with a practically negligible loss of hippuric acid. The ethyl acetate solution is washed with a few cc. of water to remove adhering salt and the combined washings are shaken with some additional ethyl acetate which is carefully separated, washed and then added to the main bulk of extract. The ethyl acetate solution is transferred to a round bottom flask and subjected to a thorough steam distillation, about 750 cc. of distillate being collected. By this means a considerable amount of volatile acids is removed. The hot aqueous solution, containing the hippuric acid, is boiled with a pinch of charcoal and then filtered and the residue washed with boiling water. When large amounts of hippuric acid are present it is well to allow this filtrate to stand for some time in a cool place in order that a large part of the hippuric acid may crystallize out directly. The filtrate from the crystals is carefully shaken in a separatory funnel with a

mixture of two parts by volume of benzene and one volume of alcohol-free ether; in this way the bulk of the small amounts of indolacetic acid and oxyphenylacetic and related acids is readily removed while the loss of hippuric acid is almost nil. The aqueous solution containing the hippuric acid is evaporated to dryness, the residue dissolved in a little boiling water and the filtered solution transferred to a weighed glass dish. Any hippuric acid crystals obtained at a previous stage are added and the whole is allowed to evaporate at a moderate temperature. The hippuric acid crystallizes out in a surprisingly pure state. The amount of uncrystallizable matter amounts to not more than about twenty milligrams and is a practically constant quantity. The hippuric acid is dried in the water-oven and weighed and its purity is then checked by a nitrogen determination. The melting point of the acid, which is only slightly discolored, is usually not more than two or three degrees below the correct value and a single recrystallization from water at once yields a pure product of correct melting point.

In the case of Experiment I, the hippuric acid weighed on the fourth, fifth and sixth days was mixed together. The melting point was 184 to 186° and the nitrogen determination gave the following results:

0.5988 gram gave NH_3 = 0.04732 g. N = 7.90 per cent
Calculated for $C_9H_9O_3N$ = 7.82 "

The examinations for free benzoic acid were based upon the following procedure: The fresh acidified urine was extracted with benzene in a continuous extractor and the benzene extract vigorously shaken with a small quantity of caustic soda solution. The benzoic acid if present, is in this way removed from the benzene solution. If much benzoic acid be present it is at once precipitated on acidifying the concentrated alkaline solution. If no precipitation occurs the solution is shaken with ether and the residue remaining, after the evaporation of the ether is then examined for benzoic acid. This method was tested by adding benzoic acid to urine and was found more satisfactory than those in general use. No benzoic acid was found in any of the specimens of urine examined in connection with the present work.

SUMMARY.

Benzoic acid taken by men in doses of 5 to 10 grams per day for two or three days, in the form of sodium benzoate, undergoes a practically complete conversion into hippuric acid and is eliminated as such in the urine. Under these conditions no free benzoic acid is excreted. There is no evidence of the conversion of benzoic acid into any aromatic oxy-acid; neither is there any evidence of any material part of the benzoic acid undergoing complete combustion in the animal body. With the doses of benzoic acid mentioned the increase in glycuronic acid derivatives in the urine is trifling. These results confirm and extend those of Lewinski and are in opposition to those of Brugsch and Tsuchiga.

An improved method for the estimation of hippuric acid is described.

A CHEMICAL AND BACTERIOLOGICAL STUDY OF FRESH EGGS.[1]

By M. E. PENNINGTON.

(Received for publication, November 16, 1909.)

A study of the eggs commonly offered for sale in public markets will readily show that variations exist between those for which the same price is charged and which are described under the same label. While literature giving the chemical analysis of the eggs of the common domestic fowls, especially the hen, is fairly plentiful, there are lacking certain data, such as the distribution of the protein forms of nitrogen in egg-white, or the usual fat numbers for the yolk, which are very desirable if comparisons of fresh, high grade eggs, and stale or low grade eggs are to be made. On the other hand, there has been much more done on the phosphorus compounds, both organic and inorganic, which occur in the yolk of eggs and on the isolation and identification of such substances as cholesterol, certain glycerides, nuclein, etc.; in other words, those substances which would tend to show the presence or absence of eggs when mixed with other materials as in noodles, and tanners' fine kid dressings.

The presence of bacteria and molds in eggs, their number, distribution and species, is a subject of economic importance as well as of scientific interest, because the keeping quality of an egg depends to a very great extent upon its bacterial content. Our knowledge of egg bacteriology is much more fragmentary than is our knowledge of its chemistry.

Hence, it seems desirable that a careful study be made of fresh eggs of known history—from both a chemical and bacteriological point of view, that there may be a basis of comparison for eggs of unknown history or doubtful quality.

[1] Presented at the Congress of Applied Chemistry, held in London, June, 1909.

CHEMICAL ANALYSIS OF EGGS.

The earliest analysis of hen's eggs recorded by König[1] is that made by Bostock of egg-white, about 1855. He determined the amount of water, nitrogen, fat and ash. These constituents have been determined a number of times since then for white and yolk separately and for the entire egg. Langworthy[2] gives the following figures which may be taken as typical.

TABLE I.

Gross Analysis of Eggs.

	IN THE NATURAL CONDITION.				
	Shell.	Water.	Nitrogen.	Fat.	Ash.
		p. c.	*p. c.*	*p. c.*	*p. c.*
Whole egg	with shell 11.2	65.5	11.9	9.3	0.9
	without shell	73.7	13.4	10.5	1.0
White		86.2	12.3	0.20	0.6
Yolk		49.5	15.7	33.3	1.1
Whole egg boiled	without shell	73.3	13.3	12.0	0.8
Raw, white shelled egg	10.7	65.6	11.8	10.8	0.6
Raw, brown shelled egg	10.9	64.8	11.9	12.2	0.7

Lebbin[3] states that of the total weight of an egg the shell is 10.89 per cent, the yolk 15.50 per cent, and the white 29.5 per cent. These figures are based on the examination of six eggs.

Cook,[4] gives the distribution of the protein nitrogen in two samples of fresh boiled eggs, whites and yolks separately. The mean of the two analyses follows:

[1] König: *Chemie der Menschlichen Nahrungs und Genussmittel*, 1. p. 98.

[2] Langworthy: Farmers Bulletin No. 128—United States Department of Agriculture.

[3] Lebbin: *Zeit. oeffentl. Chem.* vi. p. 148, 1900.

[4] Cook: U. S. Department of Agriculture, Bureau of Chemistry, Bulletin No. 115.

TABLE II.

	Nitrogen of egg present as—				
	Total.	Coagulable.	Non-coagulable.	Proteose and peptones.	Amido.
Wet Basis					
Yolk of boiled egg............	2.62	2.479	0.136	0.049*	0.092
White of boiled egg...........	1.76	1.463	0.299	0.263*	0.036
Dry Basis					
Yolk of boiled egg............	4.9538	4.6838	0.2697	0.0949*	0.1748
White of boiled egg...........	12.6946	10.5375	2.1525	1.8936*	0.2599

* Difference between uncoagulable and amido nitrogen.

The analytical methods used by Cook are those of the Association of Official Agricultural Chemists.[1] His work was done on a sample from the mixed yolks and whites of four eggs, which, according to the statement of a "reliable local dealer," were laid on the previous day.

The analyses made in this laboratory were usually on from 12 to 18 eggs, all less than 48 hours old; generally about 24 hours. They were collected from the nests at short intervals, dated, the hour of collection marked on the shell and then kept in a cool, dark place until the time for analysis. The two breeds, Barred Plymouth Rocks and White Leghorns, were kept separate. We have, therefore, an amplification of Langworthy's study of dark and light shelled eggs. The quality of the eggs, aside from the question of freshness, was of the best. The hens were young, well and regularly fed, clean, and the nests were clean. The Plymouth Rock eggs were all late summer eggs, September and October, except analysis 184, which was made on March eggs. The Leghorn eggs were early spring eggs, February and March. It is of interest to note that for these well-fed hens there is practically no difference in composition between Fall and Spring eggs. All the chemical analyses, and most of the bacteriological, were made on fertilized eggs. Some bacteriological work has been

[1] U. S. Department of Agriculture, Bureau of Chemistry, Bulletin No. 107. Revised.

done on unfertilized eggs to determine their relative content of organisms.

For the analysis of white and yolk individually it is necessary to separate the two completely. To eliminate all yolk from the white of fresh eggs is a simple matter; to remove the last traces of white which adheres to the yolk is not so simple. The following scheme, however, serves the purpose, and that it is accurate is indicated by the agreement of the analyses of the yolks given in the tables:

Crack the egg at the equator and separate the white and yolk in the usual housewife fashion, being very careful not to rupture the vitaline membrane.

When as much of the white as possible has been drained off, transfer the yolk, by gently sliding it out of the shell to a piece of clean copper gauze such as is used in the laboratory as a protection from free flames.

With a piece of filter paper absorb around the edges of the yolk, but without touching it, as much of the white as possible; and also take off in this way the white which is underneath the egg.

With a very gentle stream from a wash bottle wash the yolk. This can be done without breaking the membrane. Every trace of the white can be so removed and by very gently rolling the side of the yolk next to the copper gauze can be brought uppermost, and washed. Filter paper will absorb the excess of moisture.

Then with exceeding care the almost dry yolk can be rolled upon a piece of filter paper and the drying completed, when it is ready for analysis.

A small amount of white is lost according to this plan, but no attempt has been made in the work being presented to determine the relative amounts of white and yolk. It is the composition of each which is desired.

Water, ether-soluble material and ash in both yolk and white are determined according to the usual official methods. The apportionment of protein nitrogen in the egg-white is determined as that coagulable by heat in a solution faintly acid to litmus, and as non-coagulable by heat in the filtrate from the above. This filtrate is also studied by precipitating the albumoses with zinc sulphate and determining the amino bodies by the method of Bigelow and Cook.[1] All nitrogen determinations are made by the Gunning modification of Kjeldahl's method.

[1] Bigelow and Cook: *Journ. Amer. Chem. Soc*, xxiii, p. 1485, 1906.

For the complete coagulation of the higher proteins of egg-white it has been found advisable to dilute with twenty times its weight of water, make faintly acid with acetic acid, using litmus paper as an indicator and heat on an electric plate at about 90° C. The volume used is, in the beginning, about 100 cc. The beaker is loosely covered with a watch glass and heating continued until the volume is about 50 cc. This gentle heating causes an even distribution of the coagulum; and the cover, by keeping a moist atmosphere, prevents that which adheres to the walls of the beaker from sticking tightly. The method has been found very satisfactory.

The filtrate from the coagulum is divided into two portions, one of which serves for the estimation of the albumoses, the other for amino bodies. Neither of these determinations by the methods named can be accepted with satisfaction. The albumoses tend to separate as a very finely divided substance impossible to filter out and the amino duplicates show discrepancies which we have been unable to explain. Of course, both forms of nitrogen are present in minute quantities only. It is possible that with aged, or otherwise deteriorated eggs, such determinations would have a broader meaning. As a check on the zinc sulphate precipitate the amount of nitrogen thrown down by tannin-salt was determined on the same sample, and the sum of the non-coagulable nitrogenous constituents compared with the

TABLE III.

	Total nitrogen.	Nitrogen coagulable by heat.	Nitrogen in filtrate from coagulum.	Nitrogen in $ZnSO_4$ precipitate.	Nitrogen in tannin precipitate.	Nitrogen in filtrate from tannin precipitate.
No. 176. 12 Leghorn eggs.	1.65	1.46	0.195	0.188	0.176	0.005
	1.68	1.49	0.193	0.177	0.179	
No. 181. 12 Leghorn eggs.	A-1.70	1.59	0.112	0.145	0.132	
	B-1.71	1.64	0.109	0.052	0.062	
No. 184. 12 Plymouth Rock.	A-1.68	1.51	0.152	0.115	0.151	
	B-1.67	1.54	0.156	0.126	0.110	

amount of nitrogen found by direct determination in the filtrate from the coagulable matter. The tannin-salt should precipitate the albumose and peptone, if the latter is present. Its nitrogen content should, therefore, be as great, or greater, in quantity than the nitrogen content of the zinc sulphate precipitate. In but two out of five determinations was this the case.

The tannin-salt precipitate was washed with a mixture of tannic acid and salt of the same concentration as was used for the protein precipitation and kept cold during the washing. The filtrate was clear and there was nothing to indicate a resolution of the precipitate.

The variation in the amount of nitrogen coagulable by heat is not great, and this, when added to the non-coagulable nitrogen, closely approximates the total nitrogen as actually determined. Such figures may be accepted as reliable and probably represent the actual composition of the fresh egg white. For the further analysis of the non-coagulable proteins better methods must be devised.

The analyses of the white of the egg, as evidenced by 165 Plymouth Rock eggs and 69 Leghorn eggs shows that there is practically no difference in their composition. (See Table IV).

The analysis of the yolks of the eggs included the determination of the water, ash, ether extract and total solids. There were also determined the iodine, saponification and Hehner numbers, the acid value and the index of refraction. Certain of these constants have been determined for egg oil by Kitt[1] and Paladina and Losso;[2] and Vignon and Meunier[3] worked on the yolks of hard boiled eggs which were dried to constant weight at 100° C., then extracted with chloroform. Such a source cannot be considered to represent the fat of a fresh, unaltered egg.

The preparation of egg yolk for the determination of the constants may be accomplished in various ways. The aim should be to extract and dry as completely as possible the fat of the yolk, without altering its composition. Therefore, the heat used must be kept as low as possible and the solvent must not remove non-fatty materials. After numerous trials it was

[1] Kitt: *Chemiker Zeitung*, p. 303, 1897.
[2] Paladino and Losso: *Analyst*, clxi, 1896.
[3] Vignon and Meunier: *Collegium*, No. 128, p. 325, 1904.

decided to dry the yolk separated from the white as previously described by extracting first with absolute alcohol in a Soxhlet. The alcoholic extract was evaporated to dryness and the very small amount of solid material—which was almost fat free—was added to the main portion. This gave a dry, tough mass which was ground in a meat grinder then returned to another Soxhlet and extracted with petroleum ether having a boiling point below 60° C. until it was fat free. Ordinarily about two days were required for the alcoholic extraction and two for the petroleum ether. The petroleum ether was distilled off, and the residue dried in a vacuum over calcium chloride. The fat so prepared was almost free from inorganic matter. It did not contain the total amount of egg lecithin and the iodine number was much lower than when ether is used as a solvent, but the objectionable features of the latter were so numerous that it was soon discarded. So prepared, too, the values obtained from egg fat are comparable with the other food fats which are commonly extracted by petroleum ether.

A determination of the phosphorus content of the fat extracted according to various methods gave the following:

	Per cent of P_2O_5
Yolk, dried two days with alcohol, then extracted two days with petroleum ether	2.23
Dried two days with alcohol, extracted two days with ethyl ether, then two days with alcohol	2.95
Dried two days in steam oven, at 100°C., extracted two days with ethyl ether, then two days with alcohol	2.66

Water, solids and total nitrogen in egg-yolk show but slight variation in quantity, neither do the eggs of the two breeds vary. On the other hand, there is a greater difference in the constants, even in the iodine value which is probably the most accurate of all. This value, as well as the others recorded, was determined according to the methods of the Association of Official Agricultural Chemists. The acidity was determined by titration with standard alkali in a hot, alcoholic solution, using phenolphthalein as an indicator. The potassium chloride method was not accurate, in that the fat formed solid particles

TABLE IV.

ANALYSIS OF FRESH EGGS. LEGHORN AND PLYMOUTH ROCK BREED.

Plymouth Rock Eggs.

Number of sample.		Total solids.	Water.	Ether extract.	Ash.	Total nitrogen.	Nitrogen coagulable by heat.	Nitrogen non-coagulable by heat.	Albumose nitrogen.	Amino nitrogen.	Peptone nitrogen by difference.	Iodine number.	Saponification number.	Acid value.	Ester value.	Per cent of oleic acid. (calculated).	Hehner number.	Index of refraction.
No. 133. 9 eggs ..	White...	11.86	88.14	0.04	0.80	1.69	1.49	0.184										
	Yolk....	50.74	49.26	32.14	1.37	2.62												1.4643
No. 134. 6 eggs	White...	11.76	88.24	0.03	0.70	1.62	1.51	0.154										
Frozen.	Yolk....					2.62												1.4652
No. 137. 12 eggs ..	White...	12.80	87.20	0.02	0.45	1.82	1.63	0.167										
	Yolk....	52.33	47.67	32.18	1.31	2.69												1.4648
No. 139. 18 eggs...	White...	12.40	87.60	0.006	0.66	1.70	1.54	0.197										
	Yolk ...	50.63	49.37	31.44	1.35	2.59												1.4557
No. 141. 18 eggs...	White...	12.28	87.72	0.02	0.62	1.75	1.62											
	Yolk....	52.05	47.95	33.12	2.58	2.72						60.9	181.6				71.8	1.4627
No. 145. 18 eggs...	White...	12.72	87.28	0.016	0.71	1.68	1.55		0.076	0.004	0.06							
	Yolk....	52.35	47.65	33.08	1.39	2.76						64.2	191.1				83.58	1.4664
No. 146. 18 eggs.	White...	12.06	87.94	0.02	0.35	1.72	1.42											
Frozen.	Yolk....	51.64	48.38	32.38	1.34	2.65						66.8	173.4				73.4	1.4592
No. 147. 18 eggs. .	White...	11.96	88.04	0.05	0.47	1.69	1.55		0.053		0.09							
	Yolk....	52.47	47.53	32.96	1.62	2.72						63.8	176.0				71.71	
No. 149. 18 eggs.	White...	11.70	88.30	0.04	0.60	1.67	1.56		0.049	0.006	0.06							
Frozen..........	Yolk....	51.98	48.02	33.29	2.45	2.69						60.3	175.8				75.46	

No. 152. 18 eggs...	White...	11.10	88.90	0.010	0.70	1.69	1.48											
	Yolk....	52.99	47.01	33.33	2.00	2.67						62.2	184.2				77.05	
No. 184. 12 eggs...	White...	11.86	88.15	0.01	0.76	1.68	1.63	0.154	0.122									
	Yolk....	52.52	47.48	33.09	2.27	2.72						61.4	177.0	5.8	171.2	2.02	78.81	
Maximum..........	White....	12.80	88.90	0.05	0.80	1.82	1.63	0.197	0.122	0.006								
Maximum..........	Yolk.....	52.99	49.37	33.33	2.58	2.76						66.8	191.1				83.58	1.4664
Miminum	White....	11.10	87.20	0.006	0.35	1.62	1.42	0.154	0.049	0.004								
Minimum..........	Yolk.....	50.63	45.88	31.44	1.31	2.59						60.3	173.4				71.71	1.4557
Average..........	White....	12.05	87.98	0.024	0.63	1.70	1.54	0.171	0.075	0.005								
Average..........	Yolk.....	51.97	47.84	32.70	1.77	2.68						62.8	179.9	5.8	171.2	2.02	76.10	1.4620

Leghorn Eggs.

No. 172. 12 eggs...	White...	12.12	87.80	0.015	0.73	1.70	1.50		0.130	0.013	0.06						
	Yolk....	52.45	47.55	33.19	2.18	2.81						64.25	171.9	5.9	166.0	2.97	73.60
No. 173. 12 eggs...	White...	11.97	88.03	0.015	0.47	1.64	1.50		0.012								
	Yolk ...	52.55	47.46	33.10	1.78	2.77						64.1	183.9	6.0	177.9	3.02	78.21
No. 174. 9 eggs ..	White...	11.95	88.09	0.01	0.62	1.65	1.55		0.011								
	Yolk ...	52.97	47.07	31.63	1.87	2.83						66.7	185.5	5.9	179.6	2.97	83.63
No. 176. 12 eggs...	White...	12.62	87.39	0.015	0.70	1.67	1.47	0.104	0.005								
	Yolk....	52.64	47.37	32.90	1.40	2.79						65.5	194.9	6.15	188.8	3.09	82.2
No. 177. 12 eggs.	White..	12.19	87.82	0.02	0.73	1.71	1.57										
	Yolk ...	52.15	47.86	31.93	2.00	2.76						63.5	181.1	5.2	175.9	2.62	70.40
No. 181. 12 eggs...	White...	12.06	87.94	0.01	0.59	1.71	1.59	0.111									
	Yolk....	52.66	47.33	33.12	2.11	2.77						65.0	177.4	6.7	170.7	3.37	73.41
Maximum	White....	12.62	88.09	0.01	0.73	1.71	1.59	0.104	0.130								
Maximum.........	Yolk.....	52.97	47.86	33.19	2.18	2.83						66.7	194.9	6.7	188.8	3.37	83.63
Minimum........	White....	11.95	87.39	0.01	0.47	1.64	1.47	0.111	0.005								
Minimum	Yolk.....	52.15	47.07	31.63	1.40	2.76						64.1	171.9	5.2	166.0	2.62	73.41
Average..........	White....	12.15	87.86	0.010	0.61	1.68	1.54	0.153	0.010	0.013							
Average..........	Yolk.....	52.57	47.44	32.65	1.90	2.79						64.84	182.5	5.98	177.5	3.01	78.41

NITROGENOUS CONSTITUENTS CALCULATED ON WATER-FREE BASIS.

Plymouth Rock Eggs.

NUMBER OF SAMPLE.		TOTAL NITROGEN.	TOTAL NITROGEN COAGULABLE BY HEAT.	TOTAL NITROGEN NON-COAGULABLE BY HEAT.	ALBUMOSE NITROGEN.	AMINO NITROGEN.
No. 133. 9 eggs............	White...	14.25	12.58	1.55		
	Yolk....	4.77				
No. 134. 6 eggs. Frozen...	White...	13.86	12.84	1.31		
	Yolk....	5.02				
No. 137. 12 eggs............	White...	14.21	12.73	1.21		
	Yolk....	5.14				
No. 139. 18 eggs............	White...	13.70	12.41	1.59		
	Yolk....	5.10				
No. 141. 18 eggs............	White...	14.25	13.18			
	Yolk....	5.22				
No. 145. 18 eggs............	White...	13.20	12.10		0.597	0.031
	Yolk....	5.29				
No. 146. 18 eggs. Frozen...	White...	14.26	11.77			
	Yolk....	5.02				
No. 147. 18 eggs............	White...	14.21	12.96		0.443	
	Yolk....	5.17				
No. 149. 18 eggs. Frozen...	White...	14.26	13.32		0.418	0.043
	Yolk....	5.16				
No. 152. 18 eggs	White...	15.75	12.87			
	Yolk....	5.06				
No. 184. 12 eggs............	White...	15.17	13.82	1.39	1.09	
	Yolk....	5.41				

Leghorn Eggs.

NUMBER OF SAMPLE.		TOTAL NITROGEN.	TOTAL NITROGEN COAGULABLE BY HEAT.	TOTAL NITROGEN NON-COAGULABLE BY HEAT.	ALBUMOSE NITROGEN.	AMINO NITROGEN.
No. 172. 12 eggs............	White...	14.93	13.17		1.141	0.114
	Yolk....	5.59				
No. 173. 12 eggs............	White...	14.25	13.56		0.478	
	Yolk....	5.46				
No. 174. 9 eggs............	White...	14.72	13.84		0.922	
	Yolk....	5.43				
No. 176. 12 eggs............	White...	14.01	12.33		1.535	
	Yolk.....	5.44				
No. 177. 12 eggs............	White...	14.93	13.71			
	Yolk....	5.49				
No. 181. 12 eggs............	White...	14.96	14.08	0.936		
	Yolk....	5.50				

in the liquid and did not permit the alkali to penetrate, yielding thereby results that were too low. For example:

Method.	Acid value, per cent.	Calculated to free oleic acid, per cent.
By potassium chloride	2.7	1.36
By ethyl alcohol	5.8	2.91

BACTERIOLOGICAL EXAMINATION OF FRESH EGGS.

That perfectly fresh eggs from healthy hens may contain bacteria is a generally recognized fact. That they are sometimes sterile is also admitted. Whether the organisms enter the egg during its passage down the oviduct or whether they penetrate the shell either at the time of laying or afterward are questions on which opinions are contrary. The fact that certain pathogenic organisms characteristic of fowls, as vibros of chicken cholera, have been found in the egg argues for infection in the oviduct, as do the presence of foreign bodies, such as small insects; while the trade experience indicates that organisms can enter through the shell. A recent publication from Storrs Agricultural Experiment Station[1] states that organisms enter the egg in both ways. In the experiments described the yolk was broken by shaking and the mixed white and yolk tested. The number of organisms per gram or cubic centimeter is not given.

A more complete study of bacteria in the egg has been made by Pernot,[2] who has examined the eggs from ova the size of a pea to the perfect egg, finding organisms at every stage. He has also studied the permeability of the shell to microörganisms and finds that it is not bacteria-tight. This author has studied especially an organism which is pathogenic to chicks and which he calls "No. 9." In addition to this he finds the most common species inhabiting the hen's egg, both yolk and white, to be *B. subtilis*, *B. mycoides* and *B. mesentericus fuscus*.

It has seemed desirable to study from a bacteriological standpoint the whites and yolks separately, determining the num-

[1] Bulletin No. 55: Storr's Agricultural Experiment Station.

[2] Pernot: Investigation of the Mortality of Incubator Chicks. Bulletin No. 103, Oregon Agric. College, Experiment Station.

TABLE V.

BACTERIOLOGICAL STUDY OF FRESH EGGS. PLYMOUTH ROCK BREED.

Fertilized Eggs.

Sample.	Temperature of incubation of organisms.	White. Number of organisms per gram. Aërobes and facultatives per gram.	White. Facultatives and Anaërobes per gram.	White. Liquefiers per gram.	Yolk. Number of organisms per gram. Aërobes and facultatives per gram.	Yolk. Facultatives and Anaërobles per gram.	Yolk. Liquefiers per gram.	Species in White. From aërobic plates.	Species in White. From Wright's anaërobic plates.	Species in Yolk. From aërobic plates.	Species in Yolk. From Wright's anaërobic plates.
No. 133. Plymouth Rock A	37° C.	3	2	0	16	4	+	*B. Flüggei* (Chester)	*M. aurantiacus*; *B. flavescens* (Franklin); *B. aurantiacus*	*B. punctiformis* (Chester); *B. cuticularis* (Chester); *M. cinnabareus* (Flüggei); *M. aërius*; *Leptothrix hyalina*; *B. detrudens* (Wright)	*M. aurantiacus* (Schweter)
	20° C.	1	0	0	32	0	0			Brown mold, 33 per cent of all colonies	
	0° C.	—	—	—	—	—					
B	37° C.	0	1		7	2	+			Brown mold, 15 per cent of all colonies; *B. cuticularis*; *M. cinnabareus*	*M. aurantiacus*
	20° C.	0	3	0	0	2	0				
	0° C.	—	—	—	—	—					
No. 139. Plymouth Rock A	37° C.	1	0	13	0	—		*B. punctiformis*		*M. aërius*; *M. Danticii*; *M. fervidosus* (Adametz); *M. alvii* (Chester)	
	20° C.	11	0	2	24	0	3	*M. aurantiacus*; *Bact. Mansfieldii*		*M. tenacatis*; *M. tetragenous*; *M. candicans* (Flüggei)	
	0° C.	1	0		2	0		*M. orbicularis* (Ravenel)			

B	37° C.	2	0		20	0				
	20° C.	0	0	0	10	0	1			
	0° C.	0	0		0	0				
No. 141 Plymouth Rock A	37° C.	10	0		5	0		M. aërius; M. cinnabareus (Flügge); M. ovalis (Escherich)		Leptothrix hyalina (Migula); Bact. Mansfieldii; M. cinnabareus (Flügge); M. orbicularis (Ravenel)
	20° C.	32	8	6	13	0	0			
	0° C.	5	0		2	0				
B	37° C.	20	0		07	0				
	20° C.	55	0	2	22	0	+			
	0° C.	0	0		0	0				
No. 145 Plymouth Rock A	37° C.	5	0		5	0			M. viticulosis (Flügge)	M. aerogenes (Miller)
	20° C.	32	16	4	3	0	0		M. versicolor (Flügge); 2 coral yeasts	
	0° C.	5	0		4	0				
B	37° C.	0	14		6	0				
	20° C.	56	0	0	25	0	4			
	0° C.	0	0		8	0			M. aurantiacus	
No. 146. Plymouth Rock	37° C.	6	—		58	—	0	M. cinnabareus (Flügge); Leptothrix hyalina (Migula)		
	20° C.	40	10	0	285	0	+			M. punctiformis
	0° C.	12	0		0	0				
No. 147. Plymouth Rock A	37° C.	0	0		3	0				
	20° C.	11	0	0	8	0	0	B. cuticularis		M. orbicularis (Ravenel)
	0° C.	0	0		3	0				

TABLE V—Continued.

SAMPLE.	TEMPERATURE OF INCUBATION OF ORGANISMS.	WHITE. NUMBER OF ORGANISMS PER GRAM. Aërobes and facultatives per gram.	WHITE. Facultatives and Anärobes per gram.	WHITE. Liquefiers per gram.	YOLK. NUMBER OF ORGANISMS PER GRAM. Aërobes and facultatives per gram.	YOLK. Facultatives and Anaërobes per gram.	YOLK. Liquefiers per gram.	SPECIES IN WHITE. From aërobic plates.	SPECIES IN WHITE. From Wright's anërobic plates.	SPECIES IN YOLK. From aërobic plates.	SPECIES IN YOLK. From Wright's anaërobic plates.
B	37° C.	0	0		19	0					*M, aërius*
	20° C.	0	0	0	23	17	11				*Leptothrix hyatind* (Migula)
	0° C.	8	0		15	0		Coral yeast			
No. 149. Plymouth Rock. Frozen	37° C.	6	79		20	0				*Lepothrix hylaina* (Migula) *B. punctiformis*	*Crenothrix polyspora* (Cohn) *M. fervidosus* (Adametz)
A	20° C.	556	19	9	279	0	30	*B. Lustigii* *M. orbicularis* (Ravenel)			
	0° C.	39	0		121	0		*B. alcaligenes* (Petruschky) *M. orbicularis* (Ravenel) White yeast		*M. tenacatis* *M. lactis* *B. Lustigi*	

B	37° C.	flask b	roke		90	10					
	20° C.				310	50	0				
	0° C.				32	0					
No. 152. Plymouth	37° C.	150	0		3	0					
Rock	20° C.	30	0	0	0	0	0				
A	0° C.	15	0		0	0					
B	37° C.	10	0		20	20					
	20° C.	42	8	20	14	0	8				
	0° C.	0	0		15	0					
No. 184. Plymouth	37° C.	0	0		0	0	0				
Rock	20° C.	0	0	0	0	0					
A	0° C.		0								
B	37° C.	0	0		2	0					
	20° C.	0	0	0	2	0	0			*B. punctiformis* *M. cinnabareus* Brown mold *M. lactis*	
	0° C.										
No. 201. Plymouth	37° C.	0	0		30	0				*B. siccus* 100 per cent	
Rock egg from oviduct. Shell formed	20° C.	77	0	0	118	0	0	Brown mold 100 per cent		*B. siccus* 50 per cent Brown mold, 50 per cent	
	0° C.	0	0		0	0					

TABLE VI.

BACTERIOLOGICAL STUDY OF FRESH EGGS. LEGHORN BREED

Fertilized Eggs.

Sample.	Temperature of incubation of organisms.	White. Number of organisms per gram. Aërobes and facultatives per gram.	White. Facultatives and Anaërobes per gram.	White. Liquefiers per gram.	Yolk. Number of organisms per gram. Aërobes and facultatives per gram.	Yolk. Facultatives and Anaërobes per gram.	Yolk. Liquefiers per gram.	Species in white. From aërobic plates.	Species in white. From Wright's anaërobic plates.	Species in yolk. From aërobic plates.	Species in yolk. From Wright's anaërobic plates
N. 172. Leghorn A	37° C.	0	0		5	0				*B. punctiformis* 50 per cent; *Streptothrix chromogena* (Gasperinii) 50 per cent	
	20° C.	0	0	0	0	0	0	*M. orbicularis* (Ravenel) 33⅓ per cent; White yeast, 33⅓ per cent; *Streptothrix aurantiacus*—33⅓ per cent			
	0° C.	4	0		1	0				*M. cinnabareus* 50 per cent; *Streptothrix chromogena* 50 per cent	
B	37° C.	0	0		11	0				*M. rosettaceus* (Zimmerman) 33⅓ per cent; *Lepothrix hyalina* (Migula) 33⅓ per cent; *M. fervidosus* (Adametz) 33⅓ per cent	
	20° C.	0	0	0	0	0	0	White yeast 20 per cent; coral yeast 20 per cent; *M. orbicularis* (Ravenel) 20 per cent; *M. lactis* 20 per cent; *Streptothrix albido rossi doria* 20 per cent			
	0° C.	14	0		0	0					

No. 173. Leghorn A	37° C.	0	0		3	0		*Streptothrix chromomogena* 100 per cent		*Streptothrix aurantiacus* 100 per cent
	20° C.	4	0	0	3	0	0			
	0° C.	0	0		0	0		*B. detrudens* (Wright) 100 per cent		*Streptothrix aurantiacus* 100 per cent
B	37° C.	5	0		0	0				
	20° C.	0	0	0	3	0	0			*M. aurantiacus* 50 per cent; *M. aërius*
	0° C.	0	0		0	0				
No. 174. Leghorn A	37° C.	0	0		0	0				
	20° C.	0	0	0	1	0	0			
	0° C.	3	0		0	0		White yeast 50 per cent; White mold 50 per cent		*B. siccus* 33⅓ per cent; *B. punctiformis* (Wright) 33⅓ per cent; *Leptothrix hyalina* (Wright) 33⅓ per cent
No. 176. Leghorn A	37° C.	13	0		11	0		*B. punctiformis* 50 per cent; *Streptothrix farcinica* 50 per cent		*Streptothrix albido rossi doria* 100 per cent
	20° C.	0	0	0	5	0	0	*M. tenacalis* 100 per cent		
	0° C.	0	0		0	0				
B	37° C.	12	0		0	0		*Streptothrix farcinica* 50 per cent; *Streptothrix chromogena* 50 per cent		
	20° C.	0	0	0	0	0	0			
	0° C.	0	0		0	0				
No. 177. Leghorn A	37° C.	7	0		0	0		*M. candicans* (Flüggel) 100 per cent		*B. siccus* 33⅓ per cent; *M. candicans* (Flüggel) 33⅓ per cent; *Streptothrix* (Israel Kruse) 33⅓ per cent
	20° C.	0	0	0	14	0	0			
	0° C.	0	0		0	0				

TABLE VI—Continued.

SAMPLE.	TEMPERATURE OF INCUBATION OF ORGANISMS.	WHITE. NUMBER OF ORGANISMS PER GRAM.			YOLK. NUMBER OF ORGANISMS PER GRAM.			SPECIES IN WHITE.		SPECIES IN YOLK.	
		Aërobes and facultatives per gram.	Facultatives and Anaërobes per gram.	Liquefiers per gram.	Aërobes and facultatives per gram.	Facultatives and Anaërobes per gram.	Liquefiers per gram.	From aërobic plates.	From Wright's anaërobic plates.	From aerobic plates.	From Wright's anaërobic plates.
No. 177. Leghorn B	37° C.	0	0		4	0				*Streptothrix aurantiacus* 100 per cent	
	20° C.	0	0	0	14	0	0			*B. siccus* 33⅓ per cent *Streptothrix farcinica* 33⅓ per cent *Streptothrix chromogena* (Gasperinii) 33⅓ per cent	
	0° C.	0	0		0	0					
No. 181. Leghorn A	37° C.	0	0		0	0					
	20° C.	0	0	0	6	0	0			*M. candicans* 50 per cent *Streptothrix chromogena* 50 per cent	
	0° C.	0	0		0	0					
B	37° C.	17	0		11	0		*B. punctiformis* 33⅓ per cent *M. candicans* 33⅓ per-cent *Streptothrix farcinica* 33⅓ per cent		*Streptothrix farcinica* 100 per cent	
	20° C.	0	0	0	2	0	0			*M. candicans* 33⅓ per cent Brown mold 33⅓ per cent *M. aurantiacus* 33⅓ per cent	

No. 202. Leghorn	37° C.	0	0		4	0				*B. siccus* 100 per cent. Brown mold 100 per cent	
A	20° C.	5	0	0	2	0	0	*M. aurantiacus* 100 per cent			
	0° C.	1	0		0	0		White mold 100 per cent		*B. siccus* 100 per cent	
B	37° C.	0	0		208						
	20° C.	2	0	0	0		0	Green mold 100 per cent			
	0° C.	0	0								
No. 205. Leghorn	37° C.	0	0		6	0					
A	20° C.	0	0	0	2	0	0				
	0° C.	2	0		0	0					
B	37° C.	0	0		2	0					
	20° C.	0	0	0	12	0	0				
	0° C.	0	0		0	0					
No. 209. Leghorn	37° C.	11	0		12	0		*Leptothrix hyalina* 40 per cent; *B. punctiformis* 60 per cent		*B. ginglymus* (Ravenel) 50 per cent; *B. siccus* 50 per cent	
A	20° C.	0	0	0	2	0	0			Brown mold 100 per cent	
	0° C.	0	0		0	0					
B	37° C.	0	0		10	0				*M. cinnabareus* 100 per cent	
	20° C.	0	0	0	5	0	0			Brown mold 100 per cent	
	0° C.	0	0		0	0					
C	37° C.	3	0		12	0		*M. viticulosis* 100 per cent		*B. siccus* 50 per cent; *M. cinnabareus* 50 per cent	
	20° C.	0	0	0	0	0	0				
	0° C.	0	0		0	0					
No. 216. Leghorn	37° C.	0	0		1	0				*B. siccus* 100 per cent	
A	20° C.	0	0	0	0	0	0				
	0° C.	0	0		0	0					
B	37° C.	10	0		6	0		*B. detrudens* (Wright) 100 per cent		*B. detrudens* (Wrights) 50 per cent; Brown mold 50 per cent	
	20° C.	0	0	0	0	0	0				
	0° C.	5	0		0	0		Coral yeast 100 per cent			

TABLE VI—Continued.

SAMPLE.	TEMPERATURE OF INCUBATION OF ORGANISMS.	WHITE. NUMBER OF ORGANISMS PER GRAM. Aërobes and facultatives per gram.	WHITE. Facultatives and Anaërobes per gram.	WHITE. Liquefiers per gram.	YOLK. NUMBER OF ORGANISMS PER GRAM. Aërobes and facultatives per gram.	YOLK. Facultatives and Anaërobes per gram.	YOLK. Liquefiers per gram.	SPECIES IN WHITE. From aërobic plates.	SPECIES IN WHITE. From Wright's anaërobic plates.	SPECIES IN YOLK. From aërobic plates.	SPECIES IN YOLK. From Wright's anaërobic plates.
No. 224. Leghorn	37° C.	51	0		6	0					
A	20° C.	5	0	0	8	0	0				
	0° C.	0	0		0	0					
B	37° C.	2	0		36	0					
	20° C.	0	0	0	36	6	0				
	0° C.				0	0					
C	37° C.	2	3		0	0					
	20° C.	2	3	0	5	8	0				
	0° C.	0	0		8	0					
D	37° C.	21	0		2	0					
	20° C.	26	5	0	6	3	0				
	0° C.	0	0		6	0					
No. 222. Leghorn	37° C.	0	0		0	0					
A	20° C.	8	0	0	16	0	0				
	0° C.	0	0		0	0					
B	37° C.	0	0		8	0					
	20° C.	0	0	0	5	0	0				
	0° C.	0	0		0	0					
C	37° C.	0	0		1	0					
	20° C.	0	0	0	2	0	0				
	0° C.	0	0		0	0					

No. 210.	Leghorn	37° C.	0	0		3	0					
	A	20° C.	11	0	0	0	0	0				
		0° C.	0	0		2	0					
	B	37° C.	4	0		2	0					
		20° C.	0	0	0	2	0	0				
		0° C.	0	0		0	0					
	C	37° C.	4	0		3	0					
		20° C.	0	0	0	7	0	0				
		0° C.	0	0		0	0					
			2 c.c from each.			2 cc. from each.						
No. 187. eggs	Leghorn single	37° C.	0			1 mold						
		20° C.	0			0						
	No. 2	37° C.	1 mold			2 mold's						
		20° C.	0			0						
	No. 3	37° C.	0			0						
		20° C.	0			0						
	No .4	37° C.	0			0						
		20° C.	0			0						
	No. 5	37° C.	0			0						
		20° C.	0			0						
	No. 6	37° C.	0			2 mold's						
		20° C.	0			0						
	No. 7	37° C.	0			0						
		20° C.	0			0						
	No. 8	37° C.	0			0						
		20° C.	0			0						
	No. 9	37° C.	0			0						
		20° C.	0			0						

ber and also the kinds of organisms present and the relation which they bear, both numerically and specifically, to the temperature at which the cultures are incubated. Such information is of practical importance since the preservation of eggs by cold has become a universal method for the supplying of the market during the non-laying period.

The technique required for the study of the bacteria of white and yolk separately is simple. Because of the inaccuracy of measuring from small pipettes such viscous materials as egg yolk and white a weight basis was adopted and obtained as described below:

Scrub the egg well in clean water. Then soak in bichloride, 1 to 1000 for a few minutes. Wash off the egg with sterile water and place upright in a suitable holder. With sterile instruments crack the end and with sterile forceps remove small pieces of shell without rupturing the egg membrane below, until a sufficient space is made to introduce a sterile pipette.

Rupture the shell membrane with sterile forceps and with a sterile pipette withdraw about 2 cc. of the white. Place this in a small tared flask containing broken glass, and reweigh. Add 10 cc. of physiological salt solution and shake for ten minutes. The glass cuts the white of the egg and the solution is fairly satisfactory. Plate definite volumes as usual.

Pipette off, so far as possible, the white of the egg leaving the yolk unbroken. With a sterile wide-mouthed pipette puncture the vitaline membrane and withdraw about 2 cc. of yolk, which is placed in a tared flask and treated exactly as the white.

Equal portions from three eggs were mixed and examined at the same time, the A and B lots in Table V representing, therefore, 6 eggs of the same sample.

The media used were plain nutrient agar, litmus-lactose-agar, and nutrient gelatin. Plates were grown with and without air access that anaërobes might develop should they be present. They were incubated at three temperatures; namely, 37°, 20° and 0° C., and counts made of the colonies developing after periods of 72 hours, 2 weeks and 6 weeks, respectively. An endeavor has been made, also, to isolate and identify the different species occurring in both yolks and whites, and, as will be observed in the table, the relative proportions in which various species occur have been noted in a number of cases.

The tabulated findings show (Table VI) that organisms are usually to be found in both yolk and white. In the 57 experiments 18 had a decidedly greater number of bacteria in the yolk; 11 had the majority in the white and 21 had an almost even distribution; 7 were sterile. All the Plymouth Rock eggs except sample No. 184 were laid during September and October, and these eggs contained a greater number of bacteria than the early spring eggs, as evidenced by the Leghorn eggs and the one sample of Plymouth Rock eggs above noted. It may be that this difference in bacterial content depends on the breed and the conditions as well as the season.

The tabulated findings of the eggs of the Leghorn chickens show about the same number of organisms in the fertilized and unfertilized specimens. Whether this is a function of breed or of season remains to be determined. It is of interest to note, however, that the fertilized Leghorn eggs were from the same farm, and laid under the same conditions, as the fertilized Plymouth Rock eggs. The unfertilized Leghorn eggs were from a neighboring farm where conditions were similar, yet not exactly the same.

The number of species of bacteria found is noteworthy—36 in the 100 eggs from which the varieties were isolated. A list of them follows:

B. punctiformis
B. cuticularis
B. cinnabareus
B. Fluggei
B. flavescens
B. aurantiacus
B. alcaligenes (Petruschky)
B. siccus
B. detrudens (Wright)
B. ginglymus (Ravenel)

Bact. Mansfieldii

M. cinnabareus (Fluggei)
M. acrius
M. aurantiacus
M. Danticii
M. fervidosus (Adametz)
M. alvii
M. tenacatis
M. tetragenus
M. candicans (Fluggei)
M. orbicularis (Ravenel)
M. ovalis (Escherick)
M. viticulosis (Fluggei)
M. aërogenes (Miller)
M. versicolor (Fluggei)
M. punctiformis
M. lactis
M. Lustigii
M. rosettaceus (Zimmermann)

Streptothrix chromogena (Gasperinii)
Streptothrix aurantiacsus
Streptothrix albido roasi doria
Streptothrix farcinic
Streptothrix Israel Kruse
Crenothrix polyspora (Cohn)
Leptothrix hyalina (Migula)

Molds —Brown, white and green.

Yeasts —Coral and white.

A study of eggs which are not fresh is now in progress in this laborotory, the results of which will be published as soon as the work is completed.

The chemical analyses recorded in this paper were made by J. S. Hepburn, J. I. Burrell, and M. O. Stafford; the bacteriological work was done by E. Q. St. John and E. Witmer.

PHLORHIZIN GLYCOCHOLIA

A PRELIMINARY REPORT.

BY R. T. WOODYATT.

(From the Hull Physiological Laboratories[1] *of the University of Chicago and the Department of Medicine of Rush Medical College.)*

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From the time of von Mering's discovery of phlorhizin diabetes the attention of investigators in this field has been riveted upon the kidneys. "Phlorhizin diabetes" and "kidney diabetes" have come to be regarded as synonymous terms. How phlorhizin acts is a question for the solution of which there is, as yet, no generally accepted theory, but there exists a wide-spread belief that the one place where its effect occurs is in the kidneys. Barring those who at one time believed that phlorhizin caused a general qualitative change in the blood-sugar combination, whereby some sugar was rendered easily excretable by the normal kidneys, very few have opposed the idea implied or literally expressed that outside the kidneys phlorhizin is inert.

The chain of evidence, upon which this conception depends, is briefly as follows:

In 1888 von Mering[2] failing to detect hyperglycæmia in phlorhizinized animals in contrast to the condition found in all other forms of glycosuria suggested that the kidney was the organ affected. Thiel[3] and Kausch[4] showed that in birds, in which hyperglycæmia does not cause glycosuria, the administration of phlorhizin is nevertheless followed by appearance of sugar in the urine. Minkowski[5] found no hyperglycæmia in phlorhizinized animals from which both kidneys were removed, whereas it was to be

[1] Courtesy of Dr. S. A. Mathews.

[2] *Zeitschr. f. klin. Med.*, xiv, p. 405, xvi, p. 431.

[3] *Exp. Glykosurie bei Vögeln*, Dissertation, Königsberg, 1887.

[4] *Arch. f. exp. Path. u. Pharm.*, xxxvii, p. 274, 1896.

[5] *Ibid.*, xxxv, p. 85, 1893.

expected that if sugar had been liberated in other organs than the kidneys it would heap up in the blood when the kidney outlets were removed. To quote Loewi[1] "Die besondere Beteiligung der Niere bewiess dann in sehr origineller Weise N. Zuntz."

His experiment consisted in the direct injection of phlorhizin into one renal artery and the observation that the urine coming from the injected kidney became saccharine almost immediately whereas only after the lapse of a measurable time did sugar appear in the urine from the kidney of the other side. Stiles and Lusk[2] working with phlorhizinized dogs found that in these animals the administration of a definite weight of sugar produced an increase in the urinary glucose by an amount equivalent to that of the sugar administered. This has been quoted by some as confirming the idea that the sole primary action of phlorhizin is in the kidneys. Brodie and Cullis,[3] Pavy, Brodie, and Siau[4] and others have in recent times strongly discounted the old idea that phlorhizin merely increases the "filterability" of the blood sugar on the one hand or the so-called "permeability" of the kidneys on the other. In fact if Ringer's solution passing through a bloodless phlorhizinized kidney steadily becomes saccharine as reported, it proves that the essential process is independent of any filtration of blood sugar.

All of this work, however, has failed to divert attention from the kidneys albeit there is no proof in all of it that the kidneys monopolize the phlorhizin effect. Pflüger[5] has recognized this.

Now suppose, for example, that under the influence of phlorhizin, sugar appeared in the bile. After bilateral nephrectomy as practiced by Minkowski, what was to prevent excretion of sugar into the intestine? The experiments of Zuntz record only what occurred in the kidneys with no mention of other organs like the liver. Quantitative recovery in the urine, of sugar previously injected under the skin does not preclude the possibility that the sugar ultimately recovered may not first have been excreted into the duodenum and then resorbed lower down. Nor

[1] Von Noorden's *Handbuch der Pathologie des Stoffwechsels,* 1907, p. 813.
[2] *Amer. Journ. of Physiol.*, x, p. 67, 1903.
[3] *Journ. of Physiol.*, xxxiv, p. 224, 1906.
[4] *Ibid.*, xxix, p. 467, 1903.
[5] *Das Glykogen*, 2 Aufl., 1905, p. 513ff.

do the perfusion experiments with kidneys prove that sugar might not be obtained by perfusing livers under like circumstances.

In the course of some work in which the known fact was emphasized that practically all so-called kidney functions may be performed vicariously by certain other tissues, experiments were tried to ascertain whether or not these tissues would excrete sugar under the influence of phlorhizin. Interruptions have made immediate completion of this work impossible so that it was decided to communicate certain results in a preliminary report.

Experiment 1. Material, a 3 k. dog in which a biliary fistula had been made 3 days before by Dr. Dean Lewis. This dog's bile was collected for half an hour and tested for sugar by means of Fehling's solution. It had no reducing power. The dog then received subcutaneously 1 gram of phlorhizin in 15 cc. of warm 25 per cent alcohol after which collection of bile was resumed. The bile began to show reducing power almost immediately.

This bile diluted and mixed with yeast in a Lohnstein saccharometer gave a final reading corresponding to 0.43 per cent glucose.

A portion of bile freed from pigment as far as possible with animal charcoal and from excessive salts (by use of an excess of 95 per cent alcohol, evaporation, and extraction with absolute alcohol, evaporation, and re-extraction with water) gave upon warming with excess of phenylhydrazin in 50 per cent acetic acid a crop of insoluble yellow crystals having the characteristics of phenylglucosazon. After recrystallization and drying, these crystals melted at 204 to 205° C. From this it seems probable that the reducing substance in the bile is glucose.

Experiment 2. An 8 k. dog was given 1 gram of phlorhizin as in Experiment 1, then anæsthetized with ether. For 90 minutes all bile and all urine were aspirated from the respective bladders. Examination showed

Total bile........	25 cc.	Total sugar........	0.11 gram
Total urine	119 cc.	Total sugar	2.88 grams.

The total biliary sugar in this experiment then was $\frac{1}{26}$ of the total urinary sugar.

The conclusion reached from these experiments is that *phlorhizin causes glycocholia as well as glycosuria and that its action is not confined to the kidneys.*

Whether or not phlorhizin acts on other aggregations of cells such for example as the pancreas, the mucous and endothelial surfaces, etc., causing them to excrete sugar is under investigation and will be reported upon in a later paper.

In the issue of Pflüger's *Archiv* for May, 1909 (cxxviii, p. 118), which appeared a few weeks after the above observations had been made, Karl Grube proved by a different method that phlorhizin acts on the liver. His experiments, undertaken at the suggestion of Pflüger, consisted in perfusing the right and left lobes of tortoise livers, one with Ringer's solution alone, the other with Ringer's solution plus phlorhizin; one with Ringer's solution alone, another with Ringer's solution plus phlorhizin plus glucose, etc. After perfusion it was found in each case by quantitative determinations that the lobes which received phlorhizin lost more heavily in glycogen than the controls. Simultaneous presence of glucose and phlorhizin in the perfusion fluid caused no increase in liver glycogen such as occurred when glucose was used alone. Grube does not record whether or not the perfusion fluid brought out with it the glucose equivalent of the lost glycogen. He refrains from stating whether the synthesis of glycogen was checked by the phlorhizin or its hydrolysis disproportionately increased but inclines to the latter belief.

In this connection it may be stated that biliary fistula dogs rendered glycogen-free so far as that is possible by fasting and the steady use of phlorhizin still show glycocholia at the end of the regimen.

From this it may be assumed that the presence of sugar in the bile, like the presence of sugar in the urine under the same conditions, means a primary withdrawal of sugar from some source other than glycogen (e. g., protein). In the presence of a glycogen reserve such a loss would be made good at its expense; and it follows that the glycogen deficit seen in Grube's experiments may have been in part at least a secondary manifestation.

THE TOXICITY OF THALLIUM SALTS.

(First Paper)

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A few years ago the attention of one of the writers was called to a case in which ill effects appeared to have resulted from the use of thallium acetate as a medicinal agent in the treatment of persistent night sweats. A careful review of the available literature at that time revealed very few papers dealing with the physiological action of thallium, yet these recorded many interesting, though often conflicting, statements and thus became the incentive to the following study.

In a brief summary of the literature of the subject, mention should first be made of the work of Lamy,[1] who discovered thallium and independently identified it as a new element only a short time after Crookes[2] had found it. He was afflicted with general lassitude and a weakness of the lower limbs while engaged in an extensive study of the chemical properties of the element. Suspecting thallium as the cause he was the first to test its toxicity by dissolving five grams of thallous sulphate in milk and offering it to two young pups. These refused the milk after drinking a little of it, but by inadvertence six ducks, two hens, and a dog were allowed to partake of the remainder. Some hours later the dog became ill, was restless and refused food. During the night symptoms of distress became more prominent, the back was arched, respiration was labored and salivation was noted. The hind limbs, at first convulsive in movement, became partially paralyzed and this general condition was maintained until complete prostration and death occurred 64 hours after the fatal

[1] Lamy: *Comp. rend.*, lvii, p. 442, 1863.
[2] Crookes: *Chem. News*, iii, p. 193, 1861.

meal. On the evening of the dog's death the hens and ducks were found dead, or dying, a paralysis of the legs being a general symptom among those yet living. Finally both pups died in spite of every effort to save them. The symptoms emphasized by Lamy were weakness, abdominal pain, trembling, more or less effective paralysis of the hind legs, constipation, loss of appetite, and difficulty in breathing.

In the same year Paulet[1] expressed the opinion that thallium is a more energetic poison than lead. Grandeau,[2] noting the similarity in the symptoms of lead and thallium poisoning, made a few tests of their relative toxicity. A gram of thallous sulphate administered to a dog caused death in five days with all the symptoms of lead poisoning. One and one-half gram of lead acetate caused vomiting but in a few days the dog appeared to be normal. Soon after the publication of Lamy's observations on the toxic action of thallium, Crookes[3] expressed his doubt of its being extremely poisonous, since he had experienced no inconvenience from breathing its vapors or from taking doses of one or two grains[4] (.0648 to .1296 gram) of its salts. The sulphate colors the nails and hair as do the salts of mercury and causes the skin to become yellow and horny.

Marme[5] observed a tolerance for small amounts of the poison but claimed that the effects are cumulative. Blake[6] studied the effects of thallous preparations when injected intravenously. He injected .090 gram of the sulphate into the jugular vein of a rabbit. Blood ceased to flow into the lungs, owing, he asserts, to the pulmonary ganglions being affected, but after some seconds the circulation was restored and no further abnormal symptoms appeared. A second dose caused death. No results were observed after the injection into the carotid artery of various amounts up to .350 gram, but .750 gram caused death from

[1] Paulet: *Compt. rend.*, lvii, p. 494, 1863.

[2] Grandeau: *Jahresb. d. Chem.*, p. 256, 1863.

[3] Crookes: *Chemical News*, viii, p. 159, 1863.

[4] Through a typographical error in the *Jahresb. d. Chem.*, p. 256, 1863, the amount of thallium salts which Crookes is said to have taken with impunity is given as one or two grams.

[5] Marme: *Journ. d. pharm.*, 4e serie, x, p. 263, 1867.

[6] *Comp. rend.*, iii. p. 57, 1890.

"stoppage of pulmonary circulation." Luck[1] is reported to have conducted a series of experiments on warm blooded animals but the writers have been unable to secure a copy of the original paper or a satisfactory abstract of it. Curci[2] observed that thallium depressed the heart and circulation in frogs. General weakness, decreased sensibility, and difficulty in breathing preceded death which was ascribed to heart failure. In warm blooded animals he noted general weakness, collapse, and coma preceding death. In dogs the pulse was slower after the poison was given, although the blood pressure rose. After a dose of curare large enough to affect the heart and vasomotor centers, thallium caused a rise of pressure. A close resemblance to the alkali metals in physiological action is claimed for thallium by this author.

Thallium appears to have entered materia medica on the recommendation of Combemale[3] who cites the acetate as an effective remedy for night sweats. It was to be taken daily for not more than four days in succession in the form of a pill containing .100 gram of the salt. Not long afterward there appeared almost simultaneously two papers noting instances in which more or less serious after effects followed this treatment. Dubreuille[4] observed in one patient, a notable case of alopecia with its characteristic loss of hair. A similar action was reported at some length by Jeanselme.[5] A woman had been troubled by severe night sweats for several years. Thallous acetate was prescribed, and the patient received in three days nine pills, each containing .030 gram of thallium acetate, a total of .270 gram. The treatment was then discontinued not only because the perspiration became less profuse but also because the patient suffered severe pains in the abdomen and legs. In the course of a fortnight the hair of the head, eyebrows, and eyelashes began to fall out in large quantities although the patient was otherwise well except for a mild digestive disorder. A month later the hair was growing better, but some of the hairs were found to be con-

[1] Luck: *Dissert. Dorpat.*, 1891.
[2] Curci: *Ann. chim. et pharm.*, xii, p. 181.
[3] U. S. Dispensatory, 18th ed.
[4] Dubreuille *Soc. de l'anat. et de physiol. de Bordeaux*, 1898.
[5] Jeanselme. *Bull. soc. franç. de derm. et syph.*, p. 374, 1898.

stricted and discolored for a short distance above the scalp. There was no recovery of the hair which had fallen out and apparently many of the hair follicles had been irremediably injured. An examination of some of the hair by decomposing it with concentrated nitric acid, subjecting the resulting concentrated and neutralized solution to electrolysis, and testing the deposit spectroscopically for thallium, yielded negative results. Hallopeau,[1] thinking that thallium might be used to remove superfluous hair, rubbed a salve containing thallium acetate into the skin of a guinea pig. The treatment killed the subject but the hair on the rubbed surface was unchanged. It has been claimed[2] that injections of thallium are followed by general muscular atrophy, especially marked in the jaw and spine; also that it exerts a powerful depressant action on the heart, while baldness is induced in man.

Of late years no attention seems to have been devoted to this subject.

EXPERIMENTAL DATA.

Thallous salts prepared from metallic thallium and carefully freed from the less stable corresponding thallic compounds, were employed exclusively in this investigation. The acetate, sulphate and nitrate are all crystalline, stable in air, and freely soluble in water. In every case where the dose is recorded it is expressed as metallic thallium, calculated from the known weight of the salt used. The general course of the more important experiments is outlined in the following protocols.

I. A large white rat received subcutaneously 10 mg. of thallium as the nitrate at 10:15 a.m. The following day a notable lack of coördination in movement was observed and food was refused. Another injection of 10 mg. was given at 4:15 p.m. Soon thereafter the rat rapidly became stupid and moped in a corner. Nervous tremors began to appear, growing in frequency. Gradually the animal passed into a comatose condition, showing frequent micturition, thus passed the third day, and died during the night.

II. A second white rat was injected subcutaneously with 20 mg. of thallium at 11:30 a.m. It became stupid in a few hours; recovered position with difficulty when placed on its back; very little food taken;

[1] Hallopeau: *Bull. soc. franc. de derm. et syph.*, p. 374, 1898.

[2] Richet: Quoted by Cushny: *Pharmacology and Therapeutics*, p. 707.

increased excretion of urine marked; nervous tremors. On the following day a second injection of 25 mg. was given, after which the animal sank rapidly and died during the night.

III. A guinea pig received subcutaneously 5 mg. of thallium as the sulphate. Dose repeated on each of the following two days. No change apparent until the third day when lack of coördination was observed in the awkward use of the hind limbs. On the fourth the appetite failed, on the fifth the head began to tremble and lack of coördination was marked. Death followed during the night of the sixth day.

IV. Another guinea pig treated in exactly the same manner displayed the same symptoms in the same order, death resulting as before on the sixth day.

V. A rabbit was given subcutaneously 20 mg. of thallium as the acetate. There was no apparent change beyond loss of appetite and little disposition to move about, so another injection of 20 mg. was given on the second day. Third day: Subject stupid and showed evident weakness in hind limbs. Food was refused, the trembling of the head began to appear, and on the evening of the fourth day death ensued. Upon autopsy the lungs presented the usual appearance following death by asphyxia and the intestinal walls were slightly inflamed; the bladder was distended with urine, which gave a strong test for albumin and showed thallium weakly with the spectroscope.

VI. A second rabbit received by subcutaneous injection a dose of 10 mg. of thallium as the acetate. No change was observed and on the next day another injection of 10 mg. was given. Again no change until the third day when he moped somewhat but ate a little. The head began the characteristic trembling late in the afternoon. On the fourth day *food was refused, a semi-conscious condition supervened and death resulted* in the evening.

VII. Another rabbit was given 5 mg. of thallium subcutaneously daily four times in succession. No change was noted until the last day of injection when the familiar moping began, accompanied by loss of appetite and lack of coördination in the hind limbs. On the fifth day food was refused; on the sixth general weakness and a trembling of the head were prominent; and early on the seventh day death occurred.

VIII. A rabbit which had been used as one of several controls was given 5 mg. of thallium as acetate subcutaneously. The animal ate well and was lively until the fifth day when he appeared sluggish. The following two days noted a steady decline and a refusal of all food, death resulting on the eighth day, with the usual symptoms which had been observed with approaching death in other cases.

IX–X. This result was so surprising that two other rabbits were injected similarly with 5 mg. of the salt. No symptoms of thallium poisoning were noted after nine days of observation and the animals were released.

XI. This subject was a healthy mongrel dog—weight about 22 kg. One gram of thallium as the nitrate was administered subcutaneously at

10 a.m. Toward evening he became restless and the following morning was very sick; the hind limbs were partially paralyzed and the eyes were lusterless. As the day advanced a disturbance of respiration was plainly evident in spasmodic and labored breathing—a condition which continued until the end. Vomiting occurred late on the second day. Marked weakness, a striking lack of coördination in the hind limbs, a refusal of food and nervous tremors were features of the third day. The eyes were badly affected, much wax and mucus collecting behind the lids. Tears were seen constantly. The urine was increased notably in volume. The animal gave very little expression of pain, seeming on the contrary to be less sensitive to all stimuli. On the fourth day there was great weakness, low temperature and convulsive breathing. Death occurred during the night, about eighty hours after the dose was administered. No prominent lesions were noted on autopsy. The urine in the bladder gave a considerable precipitate of albumin, and from it also a small quantity of thallium sulphide was precipitated. Thallium was found also in the waxy secretions of the eye.

XII. A healthy Newfoundland dog, weighing 27 kg. was given .5 gram of thallium as the acetate *per os* with meat at 10 a.m. At 5 p.m. a slight weakness was noted in the hind limbs. On the second day there was very apparent loss of control of the hind limbs, which moved in a "jerky" manner when the dog walked. No albumin but a trace of thallium in the urine. Third day: Food was taken; feces, slightly colored with blood, and urine were passed with difficulty; albumin in urine; marked aphrodisiacal action. Fourth day: Hind limbs weak and partially paralyzed, playing the dog queer tricks in locomotion; urine in large quantity passed with difficulty, the animal being unable to assume his usual position in performing this function, sprawled awkwardly; appetite poor; pronounced test for albumin and slight test for thallium in urine. Fifth day: All food refused; nervous twitchings began; small amount of yellow liquid vomited. Sixth day: Food refused; conjunctivitis and dilation of pupils noted. Seventh day: Hind limbs nearly useless; respiration became labored; urine scanty. Eighth day: Little change, but visibly weaker; aphrodisiosis continued. Ninth day: Subject very weak: urinated with difficulty and passed with much straining a small amount of feces; unconscious at 2 p.m.; spasmodic breathing gradually becoming slower until 3:50 p.m. when death ensued.

XIII. A young bitch weighing about 9 kg. was caged and fed on a mixed diet of meat and cracker meal. She was placed under light ether anesthesia and injected subcutaneously with 50 mg. of thallium as the acetate, at 4 p.m. The next day a lack of coördination was plainly apparent in the hind limbs. At 3:30 p.m. another injection of 50 mg. was given. Third day, lack of coördination was striking at 9 a.m. When the subject was released to run about the room the hind legs would give way suddenly and sprawl awkwardly with every change of direction. Whole posterior region seemed greatly weakened. The dog stood with knees flexed. Appetite good. Fourth to ninth days: Same general condition main-

tained; food relished; urine increased notably in volume. Toward the end of this period, the subject became less lively and was often found lying on the back with feet in air. With the dog standing a slight pressure with the hand in the lumbar region would cause the hind legs to give way at once. Tenth day: Appetite impaired and eyes affected. During the following three days all food was refused, diarrhea was persistent, and a condition of general weakness developed. The hair and even the skin was eroded from the face in the path of the lachrymal secretions. Altogether a sorry looking subject. Fourteenth day: A little food taken; diarrhea improved, general condition better. Thence until the eighteenth day a gradual improvement was maintained, but the hind limbs were yet weak. Finally the animal became cheerful and normally hungry again and was released to trampdom as a reward for having recovered from such a serious condition. This recovery was a surprise of the course of experiments. The samples of urine collected gave pronounced tests for albumin and good tests for thallium until the animal was well along toward recovery.

Effect of repeated small doses per os. XIV. A healthy young dog was given 15 mg. of thallium as the acetate daily for thirteen days, a total of 195 mg. The hind limbs were first affected on the third day, a condition which thenceforth became rapidly more severe. The appetite weakened on the seventh day and failed entirely on the twelfth. Occasional vomiting and diarrhea appeared near the close of the dosing period. On the last day of the dosing period (thirteenth) there was great weakness and emaciation, the abdominal region was greatly constricted and the back bent. The dog was on his feet only once during the day and then got up to evacuate when an intestinal hemorrhage followed. The first signs of a disturbed respiration appeared toward evening. On the fourteenth day respiration was very irregular and labored, the animal sank into partial unconsciousness and died early on the fifteenth day. On post-mortem examination the subject was found to be greatly emaciated. Small ulcerous sores were noted on the inside of the thighs of the hind legs and on the abdomen. The gums were discolored by a purplish blue line at the base of the teeth. The gall bladder was distended with bile. The intestines were intensely congested throughout their entire length. Free blood was found in the small intestine, the whole inner wall of which was extensively inflamed. The urine began to yield tests for albumin early in the dosing period, the amount increasing as the case progressed. Tests for sugar, bile, and blood were negative. In a few samples casts of the hyaline type were observed.

XV. The subject of this test was an active and healthy dog of about 21 kg. weight. He was fed on lean meat and cracker meal for several days and then given 40 mg. of thallium as the nitrate on each of five successive days. In this case the same symptoms were manifested as in the other cases and developed in the same order,—lack of coördination in hind limbs, diuresis, loss of appetite, nervous tremors, albuminuria, vomiting, diarrhea, aphrodisiosis, respiratory disturbance and death on the fifteenth day. Post-mortem examination revealed ulcerous sores and pus sacs in

the pelvic region, the common purplish blue line around the gums, a gall bladder greatly distended and filled with bile and the unfailing inflammatory condition of the intestines. Albuminuria first appeared faintly on the fourth day and increased in severity until the twelfth when very large amounts of albumin were found in the urine.

The table on following page gives an outline of the experiments on warm blooded animals.

Experiments on cold blooded animals. A number of toads were injected with thallium acetate in single and repeated doses varying from .001 gram to .030 gram. Injections were made directly into a lymph sinus on the back, and as in the other experiments proper controls were kept under observation during the course of the tests. The results indicate that doses of .005 gram and upward are fatal to these animals, which average about 300 grams in weight. Even this usually lethargic organism, however, manifested in a very decisive way the main symptoms which marked the course of action in mammals. Loss of control of the hind limbs and death by asphyxia were always prominent, the nervous mechanism controlling the heartbeat seeming to be unaffected while that of respiration was influenced to such an extent that this became the immediate cause of death.

Two rectangular glass battery jars having a capacity of about thirteen liters were filled with fresh sea water, the bottom covered with sand and stones, and sea lettuce added to assist in keeping the water properly oxygenated. Thallous chloride to the amount of .6104 gram was dissolved in the water of one of the tanks, thus providing a 1:2500 thallium solution. A toad fish was placed in each tank. These fish (genus *Porichthys*), weighing 30 to 50 grams, are very hardy, being accustomed to withstand lack of food and great changes in the concentration of the sea water in which they thrive as a result of frequent isolation in pools between high tides. Within a few hours the respiration of the test fish became labored and spasmodic. Early on the second day its movements were sluggish and dyspnœa was marked, but as the day advanced the subject showed signs of great distress. The head was thrown violently upward and the gills worked painfully. Swimming movements were erratic and weak and the fish drifted helplessly into collision with the stones or the sides of the vessel. At noon it turned on its side

NO.	DATE	SUBJECT	SALT USED	ADMINISTERED	DOSAGE*	DAYS GIVEN	TOTAL	OBSERVATIONS
					gram		gram	
I.	Jan. 29, '08	White rat	nitrate	subcutaneously	0.010	2	0.020	Died on second day
II.	" 29, '08	" "	"	"	0.020 / 0.025	2	0.045	" " " "
III.	Feb. 4, '08	Guinea pig.	sulphate	"	0.005	3	0.015	" " fifth "
IV.	" 4, '08	" "	"	"	0.005	3	0.015	" " " "
V.	June 4, '08	Rabbit	acetate	"	0.020	2	0.040	" " fourth "
VI.	" 4, '08	"	"	"	0.010	2	0.020	" " " "
VII.	" 4, '08	"	"	"	0.005	4	0.020	" " sixth "
VIII.	" 8, '08	"	"	"	0.005	1	0.005	" " seventh day
IX.	" 14, '08	"	"	"	0.005	1	0.005	No serious effects; recovered
X.	" 14, '08	"	"	"	0.005	1	0.005	" " " "
XI.	May 28, '08	Dog (wt. 22 kg.)	nitrate	per os	1.000	1	1.000	Died on fourth day
XII.	June 3, '08	" " 27 "	acetate	" "	0.500	1	0.500	" " ninth "
XIII.	Oct. 26, '08	" " 9 "	"	subcutaneously	0.050	2	0.100	Seriously affected but recovered.
XIV.	Jan. 1, '09	" " 10 "	"	per os.	0.015	13	0.195	Died on fifteenth day
XV.	" 13, '09	" " 12 "	nitrate	" "	0.040	5	0.200	" " " "
XVI.	Oct. 3, '08	" " 9½ "	sulphate	" "	0.050	5	0.250	" " twelfth "
XVII.	" 31, '08	" " 9½ "	acetate	" "	0.030	4	0.120	" " eighth "
XVIII.	" 31, '08	" " 15 "	nitrate	" "	0.100	3	0.300	" " seventh "

and soon thereafter made a quick rush to the surface, making desperate efforts to breathe, and then fell head downward to the bottom where death occurred an hour later, and fifty hours after the beginning of the experiment. That lack of oxygen in the water was not even a contributing cause of death was shown by the normal respiration and activity of the control.

This experiment was repeated, using a toad fish and a young rock cod (genus *Cottidæ*). The rock cod died during the following night after displaying signs of embarrassed respiration wi hin a few hours after being placed in the tank. The toad fish was seriously affected at the close of the second day and though transferred to fresh sea water, the symptoms progressed unabated, death occurring 53 hours after the test began. In other experiments at a concentration of 1:1250 death resulted within 20 hours.

In order to test the resistance of fish of larger variety toward thallium salts injected into the body, a large concrete tank was filled with fresh sea water and in it were placed three bull fish (genus *Sebastodes*) and two "blue cod" (*Ophidon elongatus*). Seaweed, rocks and sand were added and a stream of fresh sea water was kept running through the tank much of the time. Mussels, which were eaten greedily, were provided as food. After being two days under observation, three of the fish were injected with thallium acetate directly into the lymphatic trunk just behind the lateral fin.

No. I. Bull fish, weight 2600 grams. Given 25 mg. thallium.
No. II. Blue cod, weight 750 grams (approx.). Given 15 mg. thallium
No. III. Bull fish, weight 1900 grams. Given 15 mg. thallium.

Second day: No I appeared to be normal. No. II was affected, being sluggish in movement. No. III was normal, and received another injection of 15 mg. Third day: No. I was less active and appeared ill. No. II was in a dying condition at 10 a.m., with the gill movements irregular and convulsive. Death followed at 2 p.m. No. III was seriously affected and gave scarcely any response when disturbed. It showed a tendency to lie on one side and swam in a circuitous path. Fourth day: No. I was visibly affected. There was a notable loss of control of direction in swimming. The fish swam repeatedly head forward against the side of the tank when trying apparently to avoid it. Ex-

haustion came on quickly after slight exertion. The body was tilted to one side in swimming and resting. No. III died at 9 a.m. Fifth day: No. I was found dead at 8 a.m. The controls were normal and active.

Discussion of results. It would appear from these experiments that thallium deserves to be classed among the most toxic of the elements, progressing in its physiological action with a remarkable certainty and definiteness. In general death has been found to result from smaller doses than any yet emphasized by previous investigators. Lamy expressed great surprise that five grams of thallous sulphate (containing 4.06 grams thallium) should kill a dog, two young pups, six ducks and two hens. In these experiments two rats, two guinea pigs, four rabbits, six toads, and seven dogs have been killed with less than 3 grams of thallium.

It is apparent that thallium stands in an entirely different class from lead, which it greatly excels in toxicity, and ranks very close to arsenic. That the latter is the more deadly however when administered in a single dose, is hardly to be doubted. Accepting .200 gram of arsenious oxide as a fatal dose for an adult, it seems highly improbable that that amount of thallium in a single dose would cause death in man, although it might be expected to give rise to a serious disturbance of the appetite and digestion, and of the power of coördinated movement in the lower limbs. One important difference in comparison with arsenic may be emphasized here and that is the absence of any apparent tolerance for the metal. The administration of a given amount of one of its salts in repeated small daily doses seems to be scarcely less effective than when given as a single dose. This tendency toward a strong cumulative action is indicated in a number of the experiments, notably Experiment XIV where .195 gram, given in thirteen daily doses of .015 gram each, produced death in the same time as .200 gram given in five doses of .040 gram each.

The symptoms displayed by all the subjects have shown a striking and consistent conformity and have developed in well defined order. The first one to appear is that of lack of coördination in locomotion, and this is shown by dogs, rabbits, toads and fishes alike. In quadrupeds this difficulty is localized in the

hind quarters, which gradually become more or less completely paralyzed. With fishes there is lack of balance, the subject swims in queer positions and is unable to guide or steer himself in the desired direction. This symptom develops very soon after the poison is given and very small amounts are enough to cause its appearance. After injection with 50 mg. one dog (XIII) displayed it 15 hours later, and another (XIV) made vague and awkward motions with his hind legs after 45 mg, had been given in three daily doses of 15 mg. each.

Among the nervous disorders a persistent trembling of the head has been noticed in all warm-blooded subjects. In some cases great dilation of the pupils was observed and general tremors all over the body were common. At times the dog shivered as though cold. The skin was always dry.

The urine is greatly increased in quantity for some days after thallium is first administered. In one dog (XVI) the average daily amount of urine before dosing was 450 cc., during the dosing period, 600 cc., and after this irregular in quantity and often lacking altogether on certain days. In another case (XVII) the normal average daily amount was 280 cc., while during the dosing period the average was 360 cc. Still another (XV) passed a daily average of 300 cc. before dosing, but this was increased to 460 cc. when thallium was given. The tear glands are likewise stimulated; several dogs wept continuously and thallium was detected in the secretion. The secretion of bile was apparently excited; in all cases where post-mortem examinations were made the bile bladder was found gorged with bile, and in the toads the livers were discolored. All the dogs vomited more or less of mucus colored with bile which has also been found in the contents of the stomach after death. The whites of the eyes in several dogs were colored yellow. Whether these conditions are due to a true cholagogic action is yet open to question. There were no evidences, however, of any increased activity in the secretions of saliva, and no symptoms of salivation were noticed. Neither was constipation induced at any stage of the poisoning, and in the dog diarrhea always occurred. The poison evidently exerts some aphrodisiacal action, since in three of the dogs the organ involved was constantly erected. Small sores and pus sacs were found on three dogs in the pelvic region. The purplish

blue line observed on the gums may be due to a deposition of thallous sulphide, since the similar blue line found in lead poisoning has been ascribed to a deposit of the sulphide of the metal.

The last symptom to appear is the difficulty in respiration. This trouble is only slightly felt at first but gradually increases, so that a short time before death the subject fights desperately for each breath. During this period the heart beats slowly but strongly. Finally, however, a climax is reached in this spasmodic gasping for breath, the subjects become quiet and all muscular effort ceases, inspirations occur at longer and longer intervals, becoming so slight as to be scarcely noticed, and finally cease altogether. In nearly all cases death has occurred very quietly and coma has preceded the end by several hours. The direct cause of death was plainly due to asphyxia induced by an inhibition of respiratory impulses. There was no indication of heart failure, and in the toads the heart was in every case found beating many minutes after breathing had ceased and when the animals were partly dissected. The labored respiration was especially striking in the case of the fishes, several subjects making the most violent motions with the head at each effort to breathe.

The albuminuria which begins a day or two after such small doses as 30 mg. in a dog has been a consistent symptom. At first the amount of albumin is very slight, but it increases in quantity until excessive amounts are excreted daily.

In the whole list of experiments it is a remarkable fact that none of the subjects gave any expression of suffering. There was no whining nor moaning heard from any of the animals. In view of the extensive and oftentimes extreme congestion of the alimentary tract this fact points to greatly diminished sensibility. In fact, all classes of subjects became dull and stupid and for some time before death were in a more or less comatose condition. In none of the injected subjects was there any local swelling or soreness at the point of injection nor any hardening of the tissues due to precipitated proteins. Neither was there any discoloration of skin or hair noticed in any subject and the skin of one of the writers was unaffected by small amounts of various salts rubbed in. Excessive loss of hair was observed in all dogs which were kept under observation for a prolonged period after the administration of thallium salts, thus bearing out a symptom already noted on human patients.

The great similarity of the symptoms in thallium poisoning to those induced by lead is evident. With both poisons the lower limbs are affected with lack of coördination and paralysis. There is the same loss of appetite, the abdominal region is constricted and there is notable congestion in the intestines. In both cases much hair is shed; and a colored line (blue with lead, purplish blue with thallium) is found on the gums. Emaciation and conjunctivitis are common to both forms of intoxication, while general nervous disorders (lead palsy) are induced by both metals.

With lead, however, constipation is the rule and sometimes this is the only symptom developed by medicinal doses. Diarrhea occurs but is rare, as is also vomiting, which usually occurs only when large doses which act as an emetic have been taken. Also in plumbic poisoning the urine is scanty and frequently suppressed. With thallium diarrhea is always present and vomiting is the rule. This last is induced and persists many days after the last dose of the poison has been given, and hence there can be no emetic-like action in these vomitings. The urine also for some days is largely increased in quantity, and contains much albumin and large sediments in which casts are not uncommon. This is usually followed by an irregular secretion and even suppression of the urine in the last stages of the disorder. Such conditions suggest a probable breaking down of the secretory mechanism of the kidneys. And in this connection it is of interest to note that the symptoms which are observed in the later stages are almost identical with those which accompany uremic intoxication. Herter[1] has described a large number of cases of which most were characterized by apathy, gradually passing into tremors, conjunctivitis, depression of temperature, severe vomiting and diarrhea, with extensive inflammation of the stomach and intestines. Dyspnœa finally appears, coma intervenes, and death results generally from respiratory failure.

These symptoms are precisely those which ultimately arise in thallium poisoning. It is significant that they do not present themselves for some time (two to five days) after the metal has been administered, and that the respiratory disturbances appear

[1] *Contributions to Medecine by Pupils of Dr. Welch*, p. 69.

only in the last days of the poisoning, while the more immediate effects, such as loss of coördinated movement in the hind limbs, apparently due to the more direct action of the thallium itself, appear very soon after the substance is administered. Yet the examination of sections of the kidney of one of the dogs (XV) showed in that case lesions hardly severe enough to produce a uremic condition.[1] Thus the idea that an artificial nephritis may be induced by thallium or that uremic intoxication was the direct cause of death in the animals experimented on, is open to question. The writers hope soon to be able to report the structural changes in the kidneys of a number of cases of thallium poisoning with a view to arriving at a positive conclusion on this point.

A later paper will present the chemical data secured in the course of this work, bearing especially upon the influence of thallium upon nitrogenous metabolism, its distribution in the organism, and its excretion.

[1] The writers wish to make here grateful acknowledgment of the assistance of Dr. William Ophuls, Professor of Pathology in the Department of Medicine of Stanford University, who kindly prepared sections and made this examination for them.

THE OPTICAL INACTIVITY OF ALLANTOIN.

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At the close of an interesting paper "Ueber die Enstehung von Fäulnissbasen"[1] D. Ackermann draws attention to the fact that, so far as known, all the numerous substances[2] derived from the putrefaction of optically active protein derivatives are themselves optically inactive. In the same paper Ackermann quotes an old statement of Pasteur to the effect that the majority of those substances which are definitely classed as excretory bodies for both the animal and vegetable organisms (urea, creatinin, uric acid, oxalic acid, etc.), are also characterized by optical inactivity.

These generalizations are certainly worthy of consideration, although it is true that the inactivity of the products of putrefaction is clearly referable to the fact that bacteria are prone to attack the $C^{*}H.NH_2.COOH$ group characteristic of protein derivatives and that the removal of this grouping results in the abolition of molecular asymmetry.

Of the numerous bodies classed as excretory substances of the animal organism there is one, namely allantoin, which contains an asymmetric carbon atom (at least one is indicated in the generally accepted formula) but so far as we know, no examination of its optical behavior has been made.[3] It was clearly desirable

[1] *Zeit. f. physiol. Chem.*, lx, p. 501, 1909.

[2] Indolpropionic acid, indolacetic acid, indolcarboxylic acid, skatol, indol, *p*-oxyphenylpropionic acid, *p*-oxyphenylacetic acid, cresol, phenol, phenylpropionic acid, phenylacetic acid, formic, acetic, propionic, butyric, valeric and caproic acids, succinic acid, methane, methylmercaptan, methylamine, butylamine, isoamylamine, phenylethylamine, *p*-oxyphenylethylamine, cadaverine, putrescine, and aminovaleric acid.

[3] Cf. however, Denicke, *Liebig's Annalen*, cccxlix, p. 278.

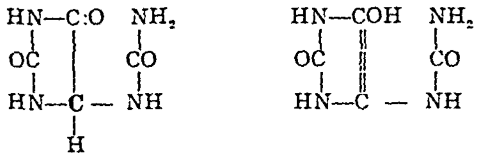
on both practical and theoretical grounds to undertake such an examination. Accordingly polarimetric observations have been made upon five different specimens of allantoin separated from urine. *In no case was any optical activity observed.* A Schmidt and Haensch triple shadow polarimeter was employed and the observations were made in aqueous solution and also in the presence of caustic potash since certain optically active structurally related substances show a much larger rotation in alkaline than in neutral solution. In order to guard against any possible racemisation in the process of separating the allantoin from urine, some experiments were made in which no other procedure than direct crystallisation was employed, but here again no optical activity was observed. It may therefore be fairly concluded that allantoin of urinary origin is optically inactive.

In order to further test the possibility of the occurrence of allantoin in optically active modifications, an attempt was made to effect a resolution of the inactive substance by bacterial action. Allantoin was dissolved in Raulin's solution in the proportion of 0.75 gram per 100 cc. and the mixture was then inoculated with a mixed culture of organisms isolated from urine undergoing active ammoniacal fermentation. These organisms were selected since it appeared probable that bacteria capable of readily decomposing urea would be most likely to effect the decomposition of a ureide such as allantoin. It was at first difficult to secure a satisfactory growth but eventually the allantoin underwent partial decomposition with the formation of much ammonia. *The residual allantoin was optically inactive.* There are few naturally occurring substances containing an asymmetric carbon atom which do not exhibit optical activity. It is therefore interesting to consider some of the possible causes of the inactivity of allantoin:

i. Optically inactive allantoin might result from its formation in the animal organism from optically inactive purin bases through the stage of inactive uric acid. This can hardly be regarded as very probable since it is more natural to suppose that the oxidases concerned would be capable of asymmetric synthesis.

ii. Allantoin may be regarded as a truly racemic compound incapable of resolution into active components by biochemical methods.

iii. It may be assumed that allantoin may exhibit tautomerism. Thus allantoin may be represented by the two following formulae, the first of which is the customary one and contains an asymmetric carbon atom while the second does not.



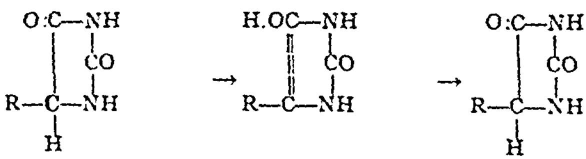
I. II.

If tautomerism of this character were proved to occur then the possibility of optical activity would be practically excluded.

The plausibility of the idea that some such tautomeric change may account for the optical inactivity of allantoin is enhanced by the fact that one of us has recently observed that the salts of a number of optically active hydantoin derivatives containing the same cyclic complex as is present in allantoin undergo rapid autoracemisation in aqueous solution at the ordinary temperature of the air. In harmony with the idea that this phenonemon is due to tautomeric change is the fact that the corresponding uramido-acids, R.CH.COOH, from which the hydantoins are derived, do

$$\begin{array}{l} \mathrm{R.CH.COOH} \\ \quad\;\;| \\ \quad\;\mathrm{NH{-}CO.NH_2} \end{array}$$

not themselves exhibit any such tendency to undergo racemisation, and in the case of these substances no labile hydrogen atom is present. The change in the case of the hydantoin derivatives may possibly be represented as follows:



(optically active hydantoin.) (inactive tautomeric form.) (optically inactive hydantoin.)

An account of these racemisation phenomena will be published shortly.

The origin of the various preparations of allantoin examined polarimetrically is indicated below.

Preparation I. Crystallised directly from urine of dog to which nucleic acid had been administered.[1] Examined in saturated aqueous solution and also in 11 per cent caustic potash solution.

Preparation II. Crystallised from urine of spleenless dog fed upon pancreas.[2] Allantoin examined in aqueous solution, 0.78 gram in 50 cc.

Preparation III. From dog's urine.[3] Allantoin crystallised in large rosettes. Examined in aqueous 1 per cent solution and also in potash solution.

Preparation IV. Allantoin separated from urine of pregnant bitch by Dr. Underhill.

Preparation V. Crystallised directly from urine of dog fed with pancreas. Examined in 0.65 per cent aqueous solution.

[1] Mendel, Underhill and White: *Amer. Journ. Physiol.*, viii, p. 395, 1903.
[2] Mendel and Jackson: *Amer. Journ. Physiol.*, iv, p. 270, 1901.
[3] Mendel and White: *Amer. Journ. Physiol.*, xii, p. 85, 1905.

THE MECHANISM OF THE OXIDATION OF GLUCOSE BY BROMINE.

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(Received for publication, December 8, 1909.)

I. INTRODUCTION.

In order to understand metabolism better the behavior of the different fuelstuffs under various conditions outside the body must first be investigated. A series of studies in this direction was accordingly started, particularly on the physico-chemical behavior of the sugars (1-7).

In a preceding investigation by Mathews and the writer (7), the following conclusions were drawn regarding the oxidation of glucose by bromine:

(*a*) Glucose behaves both as a weak acid and as a weak base. In an alkaline or neutral solution it dissociates into metal or H^+ ions and $C_6H_{11}O_6^-$ ions; in an acid solution it forms a salt of the acid by addition, yielding $C_6H_{13}O_6^+$ ions and the anion of the corresponding acid.

(*b*) Both of the glucose ions are oxidized by bromine side by side, the oxidation of the first being depressed by the addition of H^+ ions, while the other remains unaffected.

Velocity equations were worked out on this basis, taking into consideration the two kinds of glucose ions, the concentration of the H^+ and OH^- ions and of the bromine and the hypothesis was tested under very diverse conditions by measuring the rate of oxidation in the presence of various acids in different concentrations and varying amounts of bromine. The hypothesis was found to agree with all the facts observed.

In the experiments just cited the concentration of the sugar was purposely taken so large that its change of concentration was negligible. It seemed necessary, therefore, to vary also the

concentration of the sugar, to observe its rate of disappearance and to ascertain whether the equations alluded to in the above article hold true under these conditions. It was desirable also, to ascertain especially whether the oxidation of the positive glucose ions leads to the formation of gluconic acid exclusively.

The methods were the same as those in the previous investigation. The acidity developed during the reaction was determined in some of the experiments by titration with 0.1 N sodium hydroxide using phenolphthalëin as the indicator, and by subtracting the amount of hydrobromic acid formed, the amount of gluconic acid was determined.

II. DEVELOPMENT OF EQUATIONS.

It was shown in the previous paper on the subject that both the positive and negative ion are oxidizable and that in the presence of sufficiently high concentrations of H ions the acid ionization of the glucose is so depressed that only one of these two reactions needs to be considered, i.e. the oxidation of the positive glucose ion. It was shown by experiment that concentrations of H ions above 0.1 N practically completely suppress the oxidation of the negative glucose ion and in such conditions we have to deal altogether with the reaction:

$$\overset{+}{C_6H_{13}O_6} + Cl^- + Br_2 + HOH = C_6H_{12}O_7 + HCl + 2\ HBr$$

In all of the following experiments only this reaction was studied, an amount of sulphuric acid being added to make the solution 0.1 N as regards H_2SO_4.

The differential equation representing this reaction is

$$\frac{dx}{dt} = K.C_{\overset{+}{C_6H_{13}O_6}} \times C_{OH^-} \times C_{Br_2\,(free)} \cdots\cdots\cdots\cdots [I]$$

which means that the rate of the reaction will depend on three factors: (*a*) the concentration of the positive glucose ion, $C_6H_{13}O_6$: (*b*) the concentration of the hydroxylions in the solution; (*c*) the concentration of the free bromine in solution.

(*a*) The formation and quantity of the positive glucose ion

is determined by the amount of the acid present, as shown for the case of hydrochloric acid in the following equation:

$$C_6H_{12}O_6 + \overset{+}{H} + Cl^- \rightleftarrows C_6H_{13}\overset{+}{O}_6 + Cl^-$$

$$C_{C_6H_{13}O_6^+} = K'C_{C_6H_{12}O_6} \times C_{\overset{+}{H}}$$

Inasmuch as the ionic dissociation of the glucose is very small, the amount of $C_6H_{13}O_6^+$ ions will be practically directly proportional to the concentration of the glucose solution and also directly proportional to the concentration of the H ions.

(*b*) The hydroxylions are of course all derived from the water present. Their quantity is determined by the equation:

$$K\, C_{H_2O} = C_{H^+} \times C_{OH^-}$$

from which it is evident that C_{OH^-} is inversely proportional to the concentration of the H ions in solution.

(*c*) The amount of free bromine may be computed as follows:[1]

$$C_{Br_2} \times C_{Br^-} = 0.05\, C_{Br_3^-}$$

$$C_{Br\,(Total)} = C_{Br_2} + C_{Br_3^-}$$

$$C_{Br^-\,(Total)} = 2C_{Br\ Consumed}$$

$$C_{Br^-} + C_{Br_3} = 2\, C_{Br\ Consumed}$$

$$\frac{.05\ Br_3}{Br_T - Br_3} + Br_3 = 2\ Br_{Consumed}$$

$$Br_3 = \pm\sqrt{-2\,Br_{Total}\,Br_{Consumed} + \left(\frac{.05 + Br_{Total} + 2\,Br_{Consumed}}{2}\right)^2} + \frac{.05 + Br_{Total} + 2\,Br_{Consumed}}{2}$$

By subtracting this value of Br_3 from the total bromine titratable with sodium thiosulphate solution, one obtains the value for the free Br_2.

[1] See literature 7, 10, 11, 12 at the end of the paper.

By examining (*a*) and (*b*), it becomes evident at once that, provided sufficient acid is added to suppress completely the oxidation of the negative ion, the H ions hasten the reaction according to (*a*) exactly to the same extent as they retard it according to (*b*). This allowed us to neglect the concentration of the H ions provided it was over 0.1 N.

The velocity of the reaction is accordingly proportional, under these conditions, to two variables only; the concentration of the glucose and the concentration of the free bromine, and is represented by the equation

$$\frac{dx}{dt} = K\,(a - x)\,(b - x)$$

On integration this becomes

$$K = \frac{1}{t\,(a - b)} \log \text{nat} \frac{b\,(a - x)}{a\,(b - x)}$$

where K is the velocity constant, *t* the time elapsed since the beginning of the experiment, *a* and *b* the concentration respectively of the active bromine and sugar at the start, and *x* the amount of bromine and sugar consumed.

In a part of the experiments a known amount of sodium bromide solution was added, so that the concentration of the Br^- ions in the final mixture was 0.3 N. As the Br_2 in these experiments was only 0.01 N at the start, the slight increase on the concentration of the Br^- ions during the reaction due to the formation of HBr, was negligible. In the remainder of the experiments, however, where the concentration of the free bromide at the start was larger or where the reactions were so slow that it seemed undesirable to depress their rate still more by the addition of bromide the value for Br_3 had to be calculated separately for each determination. By taking the constantly changing value for Br^- in consideration we obtained a differential equation, which we were unable to integrate. For that reason the values for K were calculated for short time intervals, always using the time of the last preceding determination as a starting point. To approximate the concentration of the Br the writer added to the concentration of the HBr present at the preceding

determination (including that which is present as Br_3) one half of the HBr formed during the time interval considered and subtracted the concentration of the bromine present in the form of Br_3.

$$K = \frac{1}{t \dfrac{.05}{.05 + Br^{-}_{(\text{at start})} + \dfrac{HBr_{t_2} - HBr_{t_1}}{2} - Br_3}(a - b)} \ln \frac{b(a - x)}{a(b - x)} \quad [II]$$

The formula used in the experiment where the concentration of the Br^- ion was made .30 at the start, was

$$K = \frac{1}{t \dfrac{.05}{.35}(a - b)} \ln \frac{b(a - x)}{a(b - x)} \quad \ldots\ldots\ldots\ldots [III]$$

III. EXPERIMENTAL.

A. *To determine the constancy of K when the acidity, glucose and bromine are all varying.*

The figures given in Tables I and II indicate by the constancy of the values for K when the glucose and Br_2 are varied within wide limits, that the course of the reaction is actually controlled by those factors and in the manner stated in our hypothesis at the beginning of this paper.

B. *Is gluconic acid the sole product of the oxidation of the positive glucose ion?*

In all the experiments just quoted the change in the concentration of the bromine only was determined. The change in concentration of the sugar was assumed to be one molecule of sugar oxidized for each molecule of Br_2 used up. This assumption gave a constant value for K. To test the hypothesis further that glucose went thus into gluconic acid, it was necessary to measure at least in one experiment the degree of acidity of the mixture at varying time intervals and thus to determine the amount of gluconic acid formed. It should of course be found then that for each molecule of bromine which disappeared from the solution, one molecule of glucose has been used up and the

TABLE I.

Concentration of H_2SO_4 0.37N; *Concentration of Na Br* 0.30 N.

INITIAL CONCENTRATION OF GLUCOSE.	INITIAL CONCENTRATION OF Br_2.	NO. OF CC. OF 0.01 $Na_2S_2O_3$ REQU. TO TITRATE 10 CC. OF SOLUTION.	T (MINUTES).	K (log nat).	MEAN K (log nat).
2.00M*	0.00580M	11.60	0		
		10.68	10	0.0290	
		9.70	20	0.0312	
		8.98	30	0.0298	
		7.53	50	0.0301	
		5.80	82	0.0295	
		4.91	100	0.0301	0.0300
1.00	0.00500	10.01	0		
		9.25	15	0.0342	
		8.82	25	0.0338	
		7.89	50	0.0330	
		7.05	75	0.0310	
		6.48	100	0.0304	
		3.28	260	0.0300	0.0321
0.50	0.00520	10.40	0		
		9.29	50	0.0276	
		8.41	100	0.0298	
		7.25	165	0.0306	
		5.30	300	0.0314	0.0299
0.25	0.00516	10.32	0		
		10.00	30	0.0293	
		9.74	50	0.0312	
		9.29	100	0.0291	
		8.20	200	0.0322	
		6.83	350	0.0330	
		5.31	570	0.0328	0.0313
0.10	0.00496	9.92	0		
		6.31	1060	0.0301	
		5.44	1395	0.0298	
		1.93	3825	0.0334	0.0310
0.05	0.00515	10.30	0		
		8.37	970	0.0303	
		7.64	1185	0.0357	
		5.85	2350	0.0343	0.0335

* M refers to a molecular solution.

TABLE II.

No NaBr added. $C_H = 0.1\ N\ H_2SO_4$

INITIAL CONC. OF GLUCOSE.	TITER OF 10 CC. IN TERMS OF 0.01 N $Na_2S_2O_3$ SOLUTION.	T.	$C_{Tot.\ Br.}$	C_{Br_3}.	C_{Br^-}	$K_{(log\ nat)}$.	MEAN K.
0.77	11.01	0	0.00550				
	8.88	10	0.00444	0.00017	0.00089	0.0283	
	5.78	30	0.00289	0.00026	0.00342	0.0299	
	4.20	45	0.00210	0.00024	0.00578	0.0308	
	3.19	60	0.00160	0.00021	0.00710	0.0272	
	2.67	70	0.00134	0.00020	0.00788	0.0269	0.0286
0.55	11.02	00	0.00551				
	8.82	15	0.00441	0.00017	0.00093	0.0274	
	6.50	35	0.00325	0.00024	0.00312	0.0290	
	4.04	65	0.00202	0.00024	0.00575	0.0322	
	1.94	120	0.00097	0.00015	0.00788	0.0282	
	.81	185	0.00041	0.00006	0.00959	0.0297	0.0293
0.275	11.71	0	0.00586				
	10.40	15	0.00520	0.00010	0.00055	0.0290	
	9.04	35	0.00452	0.00022	0.00177	0.0265	
	7.14	65	0.00357	0.00029	0.00333	0.0306	
	4.85	120	0.00243	0.00028	0.00544	0.0285	
	3.05	185	0.00153	0.00022	0.00776	0.0304	0.0290
0.110	10.95	0	0.00548				
	9.38	50	0.00469	0.00010	0.00069	0.0288	
	8.05	100	0.00403	0.00021	0.00203	0.0290	
	6.72	160	0.00336	0.00025	0.00332	0.0299	0.0292
0.55	11.41	0	0.00571				
Calculated in each determination from the beginning of the experiment.	9.59	100	0.00480	0.00015	0.00076	0.0315	
	8.59	170	0.00430	0.00020	0.00121	0.0310	
	8.19	200	0.00410	0.00023	0.00161	0.0313	0.0313
	NO. OF CC. OF 0.10 N $Na_2S_2O_3$ SOL. REQU. TO TITRATE 25 CC. OF SOLUTION						
0.100	45.40	0	0.0908				
	36.45	100	0.0729	0.0066	0.0113	0.0300	
	31.50	200	0.0630	0.02187	0.0238	0.0282	
	20.30	775	0.0406	0.0263	0.0517	0.0270	
	17.00	1150	0.0340	0.0233	0.0837	0.0278	
	14.98	1480	0.0300	0.0240	0.0936	0.0268	0.0280

TABLE II—Continued

Initial conc. of glucose.	No. of cc. of 0.01 N sol. required to titrate 1 cc. of solution	T.	$C_{Tot.'Br.}$	C_{Br_3}.	C_{Br^-}	K (log nat).	Mean K.
0.15	10.00	0	0.0500				
	7.49	100	0.03745		0.01255	0.0253	
	4.30	320	0.0215	0.0110	0.0300	0.0313	0.0283
0.05	31.12	0	0.1556				
	28.50	100	0.1425		0.0131	0.0258	
	24.62	320	0.1231	0.03065	0.01495	0.0334	0.0296
Mean value of K (log nat).							0.0302*

* The cause of the divergence of this constant from the one found in the previous paper by Mathews and Bunzel, i. e., 0.0169 is twofold. There the sugar was always present in the same concentration, 0.5 M, which was regarded as unity; moreover it was thought then that the glucose used contained one molecule of water of crystallization, which was proved to be incorrect by polarimetric measurements carried on later. If the previous value of the constant is transformed on this basis, we obtain as mean value for K (log nat) from our former paper 0.0308, which agrees with the one found above.

corresponding amount of gluconic acid formed. Furthermore there ought to be for each atom of bromine transformed into hydrobromic acid, an acidity produced which is one and a half that of the HBr calculated, one-third of this acid being due to the gluconic acid formed and two-thirds of it to the HBr. The experiment was performed under the usual conditions. As the temperature was not absolutely uniform in this experiment but oscillated between 25° and 25.5°, no attempt was made to calculate the velocity constants in this experiment and only the parallelism between the changes of concentration of the two components named were studied. For this purpose at various time intervals two portions of the reaction mixture were removed from the reaction flask. One of these portions of 10 cc. volume was run into KI solution and titrated with 0.1 N sodium thiosulphate solution to determine the Br_2 used up. The other portion of 50 cc. had air blown through it for 15 minutes at room temperature to remove the Br_2. Then 10 cc. of this portion was titrated with 0.10 sodium hydrate and then with 0.1 N silver nitrate using a few drops of a saturated potassium chromate

solution as indicator to determine respectively the total acidity and the bromine in the form of bromide.

To make certain that none of the hydrobromic acid is lost during the passage of air, the following experiment was carried out: A solution of hydrobromic acid was made and air drawn through it.

Time.	Titer of 10 cc. of HBr solution
3.35	5.28 cc. 0.1 N NaOH sol.
3.55	5.30 " " " "
4.10	5.25 " " " "

From this experiment it was clear that there was no appreciable loss of hydrobromic acid.

A vigorous current of air was blown through the bromine-containing solutions in tall cylinders and by separate experiments it was determined that a complete removal of the bromine was affected.

Table III gives the results of the experiment. The strength of the sodium hydrate solution used was 0.1012 N, the silver nitrate solution was 0.09703 N, the sodium thiosulphate solution was 0.1000 N.

From the figures in Table III the following relationships become evident:

(1) The total acidity at any time in the course of the reaction determined by titration with 0.1 N sodium hydrate solution after removal of the free bromine, is, within experimental errors, equal to the acidity calculated from the amount of bromine which has disappeared from the solution assuming that each molecule of Br_2 thus used up has oxidized one nolecule of glucose to form one molecule of gluconic acid and two molecules of hydrobromic acid.

(2) Two-thirds of the acidity at any particular time is due to the hydrobromic acid formed and one-third to the gluconic acid produced.

(3) The amount of bromide found in the solution at varying times agrees closely with the amount calculated from the acidity.

(4) The amount of glucose oxidized calculated from the gluconic acid produced agrees closely with the amount calculated from the assumption that one molecule of Br_2 oxidizes one molecule of glucose to gluconic acid.

TABLE III.

$C_{glucose}$ at the start, 0.10 M; C_{NaBr}, 0.24 M; $C_{H_2SO_4}$, 0.12 N; t, 25.0° to 25.5°

I. TIME OF DETERMINATION.	II. TIME ELAPSED SINCE THE BEGINNING OF THE EXPERIMENTS. MINUTES.	III. NO. OF CC. OF .1 N $Na_2S_2O_3$ REQUIRED TO TITRATE 10 CC. OF SOLUTION.	IV. NO. OF CC. OF .101 N NaOH SOL. REQUIRED TO TITRATE 10 CC. OF SOLUTION.	V. NO. OF CC. OF .09703 N $AgNO_3$ SOL. REQUIRED TO TITRATE 10 CC. OF SOL.	VI. NO. OF CC. OF NaOH SOL. REQUIRED TO TITRATE ACID FORMED IN THE COURSE OF THE REACTION.	VII. NO. OF CC. OF $AgNO_3$ SOL. REQUIRED TO TITRATE Br FORMED IN THE COURSE OF THE REACTION.	VIII. C_H FORMED (FOUND) × N.	IX. C_H FORMED (CALCULATED FROM Br_2 CONSUMED). GLUCONIC + HYDROBROMIC ACID.	X. DIFFERENCE.	XI. C_{Br^-} FORMED (FOUND.)	XII. C_{Br^-} FORMED (CALCULATED FROM THE AMOUNT OF Br_2 CONSUMED.)	XIII. DIFFERENCE.	XIV. C GLUCOSE CONSUMED (CALCULATED FROM Br_2 CONSUMED.)	XV. C GLUCOSE CONSUMED (CALCULATED FROM ACIDITY OF SOLUTION.	XVI. DIFFERENCE.
p.m. 2.00	0	8.79	11.62	26.00	0	0	0	0	0	0	0	0	0	0.00	0.00
2.50	50	8.27	12.12	26.50	0.50	0.50	0.0051	0.0078	+0.0027	0.0048	0.0052	+0.0004	0.0026	0.0017	−0.0009
7.35	335	7.07	14.29	27.90	2.67	1.90	0.0271	0.0258	−0.0013	0.0184	0.0172	−0.0012	0.0086	0.0090	+0.0004
a.m. 8.30	1110	4.71	17.60	30.28	5.98	4.28	0.0606	0.0613	+0.0007	0.0416	0.0408	−0.0008	0.0204	0.0202	−0.0002
p.m. 8.15	1815	3.63	19.30	31.40	7.68	5.40	0.0773	0.0775	+0.0002	0.0524	0.0516	−0.0008	0.0258	0.0258	0.000
a.m. 8.50	2570	2.80	20.68	32.45	9.06	6.45	0.0916	0.0899	−0.0017	0.0626	0.0599	−0.0027	0.0300	0.0305	+0.0005
p.m. 9.15	3315	2.20	21.52	33.19	9.90	7.19	0.1002	0.0989	−0.0013	0.0693	0.0659	−0.0034	0.0330	0.0334	+0.0004

IV. DETERMINATION OF THE TEMPERATURE COEFFICIENT OF THE REACTION.

$C_{Glucose}$ 0.5 M; $C_{H_2SO_4}$ 0.3 N; C_{NaBr} 0.30 N. *Temperature* 0°.

No. of cc. of .01 N NaOH required to titrate 10 cc. of solution.	Time elapsed since beginning of the experiment.	$C_{Total\ Br_2}$.	$K_{(log\ nat)}$.
12.66	0′	0.00633	
11.50	1000	0.00575	0.00134
10.01	2430	0.00500	0.00136
7.40	5410	0.00369	0.00140
5.45	8200	0.00273	0.00144
Mean K	(log nat).		0.00139

K_{25} (log nat) 0.0302
K_0 (log nat) 0.00139
Temp. Coeff.......... .... 3.52 for 10°

V. CONCLUSIONS.

The results of the present investigation have shown: (1) that the amount of acid and of bromide produced and of glucose and bromine disappearing in the oxidation of glucose by bromine in acid solution is equal to that calculated, on the hypothesis that one molecule of bromine oxidizes one positive glucose ion to gluconic acid; and (2) that the velocity of the reaction is correctly represented by equation 1, (p. 154) as shown by the constancy of the velocity constant, K, under widely varying conditions of acidity and of different concentrations of bromine and glucose. These results strongly support the conclusions of our former paper, that glucose, like other alcohols and aldehydes, forms two series of salts; the first in which glucose behaves as an acid, the salt dissociating into metal and negative glucose ions; the second, in which glucose behaves as a base, forming an oxonium salt dissociating into $C_6H_{13}O_6^+$ ions and the negative acid radicle. Both of these ions are more easily oxidized than the neutral glucose molecule and they give rise on oxidation to different acids. The positive ion, as this research shows, goes quantitatively into

gluconic acid. The gluconic acid thus formed is presumably converted into lactone and in the course of time would be still farther oxidized by the bromine. This reaction is, however, a slow one compared to that which leads to the formation of the gluconic acid. It probably accounts for the slight tendency of the constant K to rise toward the end of the experiment, in long experiments, and in those cases where Br_2 is present in large amounts and the glucose in small amounts.

Whether gluconic acid is produced in small quantities by the oxidation of the negative glucose ion, or whether it is produced by slow oxidation also of the unionized molecule, if there is such an oxidation, cannot be said without further investigation.

The negative glucose ion when oxidized by bromine probably gives rise to the large variety of acids, described by many observers and studied particularly by Nef (13) in his splendid investigation of the transformation of the sugar molecules.

The fact that glucose oxidizes both as an oxonium salt and the salt of a metal explains the dependence of the rate and character of the oxidation on the reaction acid or alkaline of the medium. Glucose being a weak acid (14–15) dissociates into H^+ ions and negatively charged $C_6H_{11}O_6^-$ ions and in the presence of acids into positive $C_6H_{13}O_6^+$ ions and the negative acid radical. The first kind of dissociation is the principal one in the presence of alkalies.

The effect of the alkali, therefore, is to increase the number of the active negative sugar particles in solution. Acids, on the other hand, by means of the large number of H ions they furnish, greatly depress the dissociation of so weak an acid as glucose, and at the same time they form salts with the positive ion. In a neutral solution both of the oxidations of both positive and negative glucose ions go on side by side at their respective velocities, which have been determined in a former paper (7). The addition of small amounts of acid will decrease the rate of oxidation of the glucose, because, for the reason just given, acids diminish the extent of the "negative ion oxidation" which is rapid and leave unaltered the extent of the slower "positive ion oxidation." Increasing quantities of acid will slow the rate of oxidation farther and cause more and more a predominance of the latter reaction; but this decrease in rate due to increasing acidity holds true only up to a certain point i.e., to the point

where the negative ion oxidation is entirely suppressed (appr. .02 NH^+ ions); beyond that additional amounts of all acids tried, except HCl and HBr have no noticeable depressing effect. The particular action of these acids was discussed in the first communication.

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THE PURINE METABOLISM OF THE MONKEY.

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A most striking feature of the enzymes of purine metabolism is the extreme irregularity of their distribution among different organs of a single species, as well as in corresponding organs of different species. Of the many other known intracellular enzymes there are no others that have been found to exhibit such a remarkable variability of distribution. Summing up what seems to be the most reliable of the published evidence, we find that these enzymes have been found, according to *in vitro* experiments, distributed as follows:

Nuclease. Present in all cells as yet investigated.

Adenase. Present in all tissues and cells (including bacteria (4), as yet examined, except the following: the human fetus of three months (16), yeast cells (2), rabbit's liver[1] (11), the tissues of the fetal dog up to the time of birth (17), the lung of the human fetus (18), and it is but poorly developed in the adult liver of the dog (11). According to Miller and Jones (23) adenase is absent from human spleen, liver, pancreas, kidney and lung, but this statement is questioned by Schittenhelm (26).

Guanase. Present in all tissues and cells yet investigated except the spleen and liver of the pig (11, 19), the pancreas of the dog (11), and human spleen (23).

Xanthine-oxidase. Present in the spleen of the dog (11). bovine (11, 4, 6), and horse (12); absent from the spleen of man (1, 12, 23) and pig (12). Present in the liver of bovines (1). rabbit (11, 13) swine (11), and man (1, 16, 23); and possibly in the liver of the dog (31). It has also been found in the canine

[1] Schittenhelm and Schmidt (13) do not agree with this statement, finding that rabbit liver does contain adenase, but their results seem questionable.

lung and intestine (31), the bovine muscle (14), intestine (6), and lung (6, 14), but not in the bovine thymus (14) and blood (14), nor in the lung (12) and pancreas (11) of swine, the pancreas of the dog (11), the human placenta (16), nor the blood of man (9) and sheep (9). It seems to be lacking in all the chief human tissues except the liver (23, 16). It is also missing in the yeast (2) and bacterial (4) cells, and the tissues of a mollusk (*Sycotypus canaliculatus*) (22).

Uricase. If we limit the use of this term, as recommended by Battelli and Stern (10) to the oxidizing enzyme which produces allantoin and CO_2 from uric acid, and appreciate that there may possibly exist other enzymes which destroy uric acid in some other way, we find that either the kidney or the liver of every mammal investigated, except man (1, 20, 10, 15, 23) possesses uricase, while it is entirely or practically missing from every other mammalian tissue (10) and is absent from all the tissues of bird (goose) (10) and reptiles (turtle) (15). Taking what seem to be the most reliable investigations to be found in the literature we find the demonstrated presence or absence of uricolytic property to be as follows:

Liver. Present in bovine (6, 10), dog (5, 10, 15), pig (19), rabbit (13, 10), guinea pig (15), sheep (10), cat (10), horse (7, 10) dog fish (*Scyllium catulus*) (32); absent in man (1, 20, 15, 10, 23), turtle (15), goose (10, 30) and mollusk (22).

Kidney. Present in bovine (5, 6, 10), dog (slight) (10) horse (8, 10), rabbit (13); absent from the kidney of man (5, 10, 23) and sheep (10).

Muscles. Present in bovine (6, 14), and in the horse (slight) (10); absent in muscles of sheep, dog and bovine (10).

Uricolysis has been found wanting in the spleen of bovine (6, 10), dog (3, 10), pig (3, 11), sheep (10), but feebly present in the spleen of the horse (10); it is also lacking in the lung of bovine (6), horse (10) dog (10), and sheep (10); the bovine intestine (6) and the blood of man (9), bovine (10), dog[1] (10), sheep (9, 10), horse (10); the pancreas of the horse (7, 10) the brain of the horse (7) and dog (10), and the leucocytes and bone marrow of the dog (15).

[1] Preti (21) states that dog's blood is actively uricolytic.

Measuring the amount and activity of the uricase by the amount of carbon dioxide produced from uric acid by the action of tissues, the relative uricase content of different tissues in decreasing order is as follows (10): Beef kidney, horse liver, cat liver, dog liver, rabbit liver, horse kidney, and sheep liver; beef liver and dog kidney have only slight uricolytic power, and the other tissues have no distinct activity. The fresh tissues seem to contain inhibiting substances which can be removed by alcohol, and the relative activity of such alcohol treated tissues is not in quite the same order as that given above.

Although there is not complete agreement among different observers on all the data used in the above compilation, yet the agreement is on the whole fairly good, and indicates that this irregularity of distribution actually exists. On the other hand, it must be borne in mind that the absence of a given enzyme action by extracts of an organ during *in vitro* experiments proves neither the absence of the enzyme nor the inactivity of the tissue when in the living body. It is undoubtedly true that inhibiting substances may play an important part in suppressing the action of an enzyme, and in the case of the purine enzymes evidence to this effect has been furnished by Künzel and Schittenhelm (24) who found that extracts of bovine spleen prevented the destruction of uric acid by bovine kidney; and by Battelli and Stern (10) who found that in many instances the uricolytic action of tissues was much more vigorous after the tissues had been subjected to the action of alcohol, which seemed to eliminate some inhibiting agent. *In vitro* experiments also fail to reproduce *in vivo* conditions, where rapid circulation and possible co-enzymes and activators may play an indispensable rôle in bringing out the full enzyme action, as witness the futility of the attempts to reproduce with tissue extracts the evidently vigorous sugar-destroying activity of the living body.

Appreciating the limitations of *in vitro* experiments, and accepting the evidence drawn upon above for nothing more than what it actually is, we find good agreement on the point that the extracts of human tissues differ decidedly from the tissues of the other mammals in their action upon purines, and especially in respect to their ability to destroy uric acid. According to the results of the experiments of Wiechowski (20), Künzel and

Schittenhelm (1), Battelli and Stern (10), Miller and Jones (23), and Wells and Corper (15, 16), extracts of human tissues at no time exhibit any demonstrable activity in destroying uric acid under the same conditions that permit extracts of tissues of other mammals to destroy quite large amounts of uric acid in a few hours. Leaving out of the question the significance of these results for our interpretation of human purine metabolism, which has been discussed recently by Wiechowski (25), Miller and Jones (23), Schittelhelm (26), we note that man is in this respect different from every other mammal as yet investigated, including in this list dog, horse, cow, cat, guinea pig, rabbit and sheep.

Also in respect to his power to oxidize xanthine into uric acid, man is not well developed, only the liver seeming to have active powers in this respect (23), and this enzyme appearing later in fetal life than the other purine enzymes (16). Having the power to form uric acid but not to oxidize it, the human tissue extracts resemble those of the bird and reptile, but differ from those of all other mammals. Certain other experiments have suggested that the development of the purine enzymes in the ontogeny of the mammals follows the same order as in their philogenetic development, a fact of some biological significance. In this connection it has seemed desirable to follow out the same studies of the purine enzymes in the lower primates as have been made with the other mammals, in order to complete as far as possible the developmental side of the study of purine metabolism. In all but one of these experiments the material came from the common Indian monkey, *Macacus rhesus*; unfortunately no material from the higher apes was available.

One of the chief results of these observations is the demonstration that in respect to uric acid destruction by tissue extracts, the monkey is not like man, but actively destroys uric acid like the rest of the mammals. This power of uricolysis *in vitro* by extracts of monkey tissues is demonstrated by the following experiments:

Experiment I. Tissues obtained from a small monkey, which was killed under anæsthesia. The separate organs were ground fine, extracted over night at room temperature in toluene water, strained, and the extracts then allowed to act for 46 hours upon uric acid or xanthine at 40°, with a

rapid current of air running through the mixture. The analysis was performed according to the Krüger and Solomon method, the uric acid being purified from sulphuric acid according to Horbaczewski's method.

The results were as follows:

TISSUES	PURINE ADDED	RECOVERED
1. Liver (30 grams)	0.125 g. uric acid	0.0
2. Liver (30 grams.)	0.123 g. xanthine	no uric acid; trace of xanthine.
3. Kidneys (11 grams.)	0.125 g. of uric acid	0.106 g. uric acid, or 85 %.
4. Viscera (27 grams.) (heart, lungs, spleen, and thymus)	0.125 g. uric acid	0.076 g. uric acid, or 61 %.
5. Intestine (65 grams.)	0.125 g. uric acid	0.123 g. uric acid, or 98 %.
6. Intestine (65 grams)	0.123 g. xanthine	0.120 g. xanthine, or 98 %.

EXPERIMENT II (26). Large *Rhesus* monkey, dying from pulmonary tuberculosis. Liver weighed 245 grams, and the extract from this was divided into three equal portions. One was allowed to act upon 0.170 grams of uric acid in the presence of an air current for 22 hours, at the end of which time no demonstrable quantity of uric acid or of any other free purine could be found. The second portion was treated in the same way, but at the end of 22 hours autolysis with air current, the extract was placed in a tightly stoppered bottle and left 72 hours in the incubator, in order to see if, in the absence of oxygen, resynthesis of the uric acid would take place, as described by Ascoli and Izar (27). At the end of this time, however, analysis showed the presence of mere traces of free purines, not uric acid, and apparently derived from the autolysis of the liver tissue after the air had been shut off. The third part was boiled for fifteen minutes before adding the uric acid, to serve as a control, and from this solution 0.155 grams of uric acid, or 91 per cent, was recovered.

EXPERIMENT III. Small *Rhesus* monkey, killed by exposure to cold. The liver weighed 34 grams, and to an emulsion of this was added 0.210 grams of uric acid, autolysis with air current being continued 40 hours. At the end of that time no appreciable amount of purine could be precipitated from the solution.

EXPERIMENT IV. A *Rhesus* monkey, which died from acute peritonitis. To 35 grams emulsionized liver added 0.147 grams of uric acid, and digested 40 hours with an air current. At the end of this time recovered 0.057 grams of uric acid, or 38 per cent. The failure of complete destruction of uric acid by this liver may be ascribed to the effect upon the liver of the acute peritonitis from which the animal died.

In several other experiments, to be cited below, the other viscera of monkeys have been allowed to act upon xanthine in the presence of an air current without destroying it, thus supporting the evidence of the first experiment that in the monkey only the liver is actively uricolytic. That the monkey does have

power to destroy uric acid is in agreement with the finding of allantoin by Wiechowski (25) in the one sample of monkey urine which he examined. In 100 cc. of monkey urine he found 0.10 gram of allantoin, and no uric acid, the animal not being on a flesh diet. I have made a few analyses of monkey urine with the following results:

From the bladder of a large *Rhesus* monkey, which died of tuberculosis, 150 cc. of a clear, normal appearing urine was obtained, which contained a trace of albumin. One 75 cc. sample was analyzed for allantoin according to the method of Poduschka (28), but none was found. The remaining 75 cc. was concentrated to 15 cc., acidified and let stand in a cold place, but only an amorphous precipitate formed. It was then precipitated in the usual way with copper sulphate and sodium bisulphite, and from this precipitate about 10 mg. of typical uric acid crystals, giving the murexide test, was obtained.

A specimen amounting to 500 cc. was collected in toluene during several days from two monkeys, one normal and one thyroidectomized. Two samples of 100 cc. each were examined for uric acid, but none could be obtained, and the total purine precipitate was very small, containing but about 2 mg. nitrogen per 100 cc.

Several attempts were made by both Wiechowski's method and Poduschka's method to obtain allantoin, and although there was obtained a small precipitate at the stage of the procedure where the allantoin should be precipitated, yet repeated attempts to secure recognizable crystals of allantoin were unsuccessful. That there was nothing in the monkey urine which interferes with the method, and that the method itself was not at fault, was demonstrated by analyzing a mixture of monkey urine and an equal quantity of dog urine which contained a known amount of allantoin; in this experiment the theoretical quantity of allantoin was recovered.

These analyses demonstrate at least that monkeys excrete very little purine indeed, which agrees with Wiechowski's observation that those animals those tissues are uricolytic *in vitro* show a low excretion of urinary purine nitrogen; however, my attempts to demonstrate the presence of allantoin in the urine as a result of purine destruction did not give satisfactory results. The finding of a small quantity of uric acid in the urine of a monkey dying of tuberculosis is not as significant as it would be in a normal animal, since during the last hours of the sickness

the oxidative power of the liver was probably much impaired. In this connection it may be mentioned that Spiegelberg (29) quotes Pohl as having found urate deposits in the kidney of a young monkey ("Affe," kind not stated), a few weeks old.

Xanthine-oxidase seems to be absent from all the tissues of the monkey except the liver, as shown by Experiment I, in which xanthine added to intestine extract was recovered almost quantitatively after 46 hours autolysis with active air current, and by the following experiments:

EXPERIMENT V. Kidneys and spleen from a large *Rhesus* monkey were emulsionized, and each allowed to act for 22 hours upon 0.150 gram of xanthine, with an air current. At the end of this time 0.1265 gram of xanthine was recovered from the kidney extract, and 0.143 gram from the spleen extract. No uric acid was present in either extract.

EXPERIMENT VI. A small *Rhesus* monkey was killed by bleeding. Took all the viscera exclusive of the liver and intestine (total weight 44 gms.), emulsionized, and let act 40 hours upon 0.207 gram of xanthine at 40° with an active air current. At the end of this period recovered 0.1991 gram of xanthine, 96 per cent of that originally added, and no uric acid.

In view of the fact that the liver of the monkey destroys xanthine upon which it acts in the presence of abundant air, it is to be presumed that it contains xanthine-oxidase, since the route of destruction of xanthine, so far as we now know, is via uric acid.

EXPERIMENT VII. In one experiment 0.139 gram of hypoxanthine (0.233 gram hypoxanthine nitrate) was added to 65 grams of emulsionized liver in 300 cc. of water and autolysis continued under a thick layer of toluene for 17 days. At the end of this period the hypoxanthine had been about half converted into xanthine, there being recovered 0.072 gram of xanthine and 0.071 gram of hypoxanthine, but no uric acid.

Other experiments in which liver extracts have acted upon adenine, or upon adenine and guanine with very little air present, have shown a similar tendency for the newly formed hypoxanthine to go over into xanthine. (See Experiment X). Other observers have described the same change of hypoxanthine into xanthine under partially anaërobic conditions, by extracts of organs which are known to have active xanthine-oxidase when acting in the presence of abundant supply of air, and the reaction has been ascribed to the xanthine-oxidase.

In view of the known specificity of enzymes, however, it would seem more probable that the oxidation of hypoxanthine into xanthine is accomplished by a different enzyme from that which oxidizes xanthine into uric acid, especially since we have evidence that another distinct enzyme is required to oxidize the uric acid. In a number of experiments, especially in some work done with Mr. H. J. Corper upon dog spleen, which is very active in oxidizing xanthine to uric acid, I have observed this oxidation of hypoxanthine into xanthine under partially anaërobic conditions, without the demonstrable conversion of any of the xanthine into uric acid. If both reactions were accomplished by one enzyme, which was prevented by paucity of oxygen from completing the oxidation to uric acid, we should expect to find that at least part of the xanthine would be oxidized to uric acid. It is, of course, possible that the hypoxanthine is so much more easily oxidized than the xanthine that all the available oxygen is utilized for this purpose, and thus the xanthine escapes; but the presence of two distinct enzymes, one of which can act only upon xanthine, and in the presence of abundant oxygen supply, while the other can oxidize hypoxanthine even with very little free oxygen, seems more probable from the standpoint of analogy with other enzymatic actions. In this case we should need to speak of hypoxanthine-oxidase as well as xanthine-oxidase. In his most recent publication Schittenhelm (26) also mentions the possibility that it may be necessary to separate the so-called xanthine-oxidase into two enzymes, one of which acts upon xanthine and the other upon hypoxanthine, but he advances no evidence for this position.

The presence of a nuclease in monkey tissues is shown by the following experiment:

Experiment VIII. The entire viscera, except the liver, of two monkeys, together with a considerable amount of muscle, to a total weight of 368 grams, was allowed to autolyze under toluene at 40° for 18 days. At the end of this time the coagulable and insoluble materials were removed, and the free purines were isolated and identified. There was found no free uric acid, guanine or adenine; but there was recovered 0.226 gram of xanthine and 0.147 gram of hypoxanthine.

This experiment also indicates the presence of the deamidizing enzymes, adenase and guanase.

Adenase and guanase seem to be widely distributed in the tissues of the monkey, as shown by the following experiments:

EXPERIMENT IX. A long-tailed South American monkey, probably *Cebus apella*, which died from exposure—no anatomical changes being observed at autopsy. The liver weighed 80 grams, and after being emulsionized it was placed in toluene water with 0.217 gram of adenine hydrochloride (0.162 gram adenine) and 0.153 gram of guanine hydrochloride (0.117 gram guanine). The mixture was autolyzed under toluene, without air, for eleven days. Analysis of the soluble purines in the usual way showed the absence of uric acid and guanine, and there was recovered 0.104 gram of xanthine, 0.065 gram of adenine, and 0.064 gram of hypoxanthine.

The heart, lungs, kidneys, adrenals, spleen and thymus, weighing in all 43 grams, were ground up together and allowed to act upon the same amounts of adenine and guanine. From this autolysis were recovered 0.084 gram of xanthine and 0.091 gram of hypoxanthine, but no adenine, guanine or uric acid.

EXPERIMENT X. Normal *Rhesus* monkey, killed by bleeding. An emulsion of the liver, which weighed 50 grams, was allowed to act upon 0.142 gram of guanine and 0.149 gram adenine at 38° for eight days without air current. Analysis yielded 0.163 gram xanthine and 0.092 gram hypoxanthine, but no appreciable amounts of guanine, adenine or uric acid.

EXPERIMENT XI Normal *Rhesus* monkey, freshly killed, furnished 65 grams of liver tissue, which acted upon 0.146 gram of adenine hydrochloride (0.109 gram adenine) for nine days, as in the previous experiments. At the end of this time there was no appreciable amount of any purine present except hypoxanthine, of which 0.140 gram was recovered

The remaining viscera united weighed 75 grams, and these acted upon 0.109 gram of adenine and 0 137 gram of guanine. After the autolysis there was found no guanine or adenine, but 0.102 gram of xanthine, and 0 102 gram of hypoxanthine was recovered.

From these experiments it seems certain that the liver and other viscera of the monkey are able to deamidize guanine and adenine. In only one experiment (IX) was the deamidization of adenine less than total, and in this case about one-half the adenine added to the liver extract was changed to hypoxanthine. Whether this deficient activity was due to the fact that this animal died from exposure, or was due to its being of a different variety from the other monkeys examined, or was due to some individual peculiarity, cannot be said, beyond that there were no histological

changes found in the liver. It occasionally happens that individual specimens do not show the usual behavior in these experiments, which may be the reason why not all observers agree in the results they report.

It is not possible to compare the monkey with man in respect to adenase and guanase, since there is at present a strange disagreement among reports by different observers in regard to the occurrence of these two enzymes, especially adenase, in human tissues. This disagreement can hardly be due to errors of observation, for adenine cannot easily escape detection and good quantitative recovery by the usual methods of analysis, and furthermore, the fact of its deamidization is controlled by the determination of the hypoxanthine that it yields. According to Miller and Jones (23) adenase is missing from all human tissues, whether fetal or adult. Guanase, they state, is present in the liver, lung and kidney, but not in the spleen. Schittenhelm and Schmidt (18) reported the deamidization of guanine by human fetal liver, muscle, lung, intestine and spleen; and adenine was deamidized by the kidney, muscle and perhaps the liver, but apparently little if any by the lung and intestine. Schittenhelm (26) later reports that guanine is deamidized by human liver, intestine, lung and kidney, but the results with spleen were not conclusive; adenine was deamidized by human lung, and in a limited degree by the intestine and kidneys, but most of the adenine could be recovered from extracts of liver and muscle. He notes that adenine is less vigorously attacked by human tissues than is guanine. Wells and Corper (16) found guanase present in human fetuses as early as the third month, and at all later periods, while adenase was not present at the third month but appeared after the fifth month. It was found in the liver of the fetus at the fifth month, as well as in the other tissues. Adult organs were not examined, but the full term placenta when autolyzing was found to convert its guanine and adennine into xanthine and hypoxanthine. More recently I have examined the liver of another five months human fetus, which acted upon 0.081 gram of adenine for 18 days; at the end of this time 0.073 gram of hypoxanthine and 0.014 gram of adenine (0.040 gram of adenine picrate) were recovered. This corroborates the previous experiment indicating that adenase appears in the human liver before the fifth month of intrauterine life.

It is possible that the more positive evidence of adenine destruction obtained by us as contrasted with the negative results of Jones and the slight activity observed by Schittenhelm with human tissues, is due to the fact that in our experiments autolysis was continued for from 12 to 50 days, while the other authors in most cases continued the autolysis but 2 to 6 days.[1] This explanation, if found to be correct, agrees with Schittenhelm's view that human tissues can deamidize adenine *in vitro*, but that they have but a relatively slight degree of activity upon this purine as contrasted with their action upon guanine. That Schittenhelm found more evidence of adenase than did Jones may be due to the fact that his autolysis was carried out with a current of air, which probably favored more rapid deamidization by the constant stirring thus produced, and possibly by some destruction of inhibitory substances. So far as the living human organism is concerned there is no evidence of any inability to deamidize adenine, since, as Schittenhelm points out, the urine contains no considerable quantity of adenine even on a heavy nucleoprotein diet, and adenine gout has never been observed comparable to guanine gout of swine, which animals are deficient in their ability to deamidize guanine.

SUMMARY.

The liver of the monkey (*Macacus rhesus*) possesses the power of destroying uric acid *in vitro*, in the presence of an abundant supply of air. No other tissue was found to be uricolytic. Therefore, in respect to uricolytic power the monkey resembles the lower mammals rather than man. Man still remains the only mammal yet investigated whose tissues are unable to destroy uric acid *in vitro*.

The liver of the monkey can also destroy xanthine and other purines in the presence of abundant air supply, and therefore presumably possesses xanthine-oxidase. No other tissue was found to contain xanthine-oxidase. In this respect the monkey resembles man.

[1] However, in one experiment with adult human liver Miller and Jones recovered 93 per cent of the added adenine after 25 days digestion.

Hypoxanthine is converted into xanthine by liver extracts when the air supply is poor; this reaction may be accomplished by a special enzyme, hypoxanthine-oxidase, or by the xanthine-oxidase itself.

The liver and the combined viscera of the monkey seem to contain both adenase and guanase. Nuclease is also present in the tissues of the monkey.

The urine of monkeys contains very little purine nitrogen. Allantoin is probably present but was not found in any considerable amount. A small amount of uric acid was found in the urine of a monkey dying of tuberculosis, but was not present in the urine of normal monkeys.

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(1) Künzel and Schittenhelm: *Zentralbl. f. Phys. u. Path. d. Stoffwech.*, iii, p. 721, 1908.
(2) Straughn and Jones: *Journ. Biol. Chem.*, vi, p. 245, 1909.
(3) Schittenhelm: *Zeitschr. f. physiol. Chem.*, xlvi, p. 354, 1905.
(4) Schittenhelm: *Zeitschr. f. physiol. Chem.*, lvii, p. 21, 1908.
(5) Wiechowski and Wiener: Hofmeister's *Beiträge*, ix, p. 247, 1907.
(6) Künzel and Schittenhelm: *Zeitschr. f. exp. Path. u. Ther.*, v, p. 389, 1908.
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(8) Brugsch and Schittenhelm: *Zeitschr. f. exp. Path. u. Ther.*, v, p. 406, 1908.
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(10) Battelli and Stern: *Biochem. Zeitschr.*, xix, p. 219, 1909.
(11) Jones and Austrian: *Zeitschr. f. physiol. Chem.*, xlviii, p. 110, 1906.
(12) Schittenhelm: *Zeitschr. f. physiol. Chem.*, xlvi, p. 354, 1905.
(13) Schittenhelm and Schmidt: *Zeitschr. f. physiol. Chem.*, l, p. 30, 1907.
(14) Wiener: *Arch. f. exp. Path. u. Pharm.*, xlii, p. 375, 1899.
(15) Wells and Corper: *Journ. Biol. Chem.*, vi, p. 321, 1909.
(16) Wells and Corper: *Journ. Biol. Chem.*, vi, p. 469, 1909.
(17) Jones and de Angulo: *Proc. Amer. Soc. Biol. Chem.*, i, p. 193, 1909.
(18) Schittenhelm and Schmidt: *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 428, 1907.
(19) Mendel and Mitchell: *Amer. Journ. of Physiol.*, xx, p. 97, 1907.
(20) Wiechowski: *Arch. f. exp. Path. u. Pharm.*, lx, p. 185, 1909.
(21) Preti: *Zeitschr. f. physiol. Chem.*, lxii, p. 354, 1909.
(22) Mendel and Wells: *Amer. Journ. of Physiol.*, xxiv, p. 170, 1909.
(23) Miller and Jones: *Zeitschr. f. physiol. Chem.*, lxi, p. 394, 1909.
(24) Künzel and Schittenhelm: *Zeitschr. f. exp. Path. u. Ther.*, v, p. 393, 1908.

(25) Wiechowski: *Biochem. Zeitschr.*, xix, p. 368, 1909.
(26) Schittenhelm: *Zeitschr. f. physiol. Chem.*, lxiii, p. 248, 1909.
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(32) Scaffidi: *Biochem. Zeitschr.*, xviii, p. 506, 1909.

THE EFFECT OF CASTRATION ON THE METABOLISM.[1]

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This research was undertaken because no complete metabolism experiments of this kind have been carried out on healthy adult animals to determine the effect of castration on the metabolism, and because clinical evidence, supported to a certain extent by metabolism experiment, seemed to indicate that removal of the ovaries does cause at least a temporary change in the metabolism in osteomalacia. It seems to be the general opinion that castration leads to a change in the general metabolism such that there is a decrease in catabolic processes. This opinion is based on the common knowledge concerning the effect of castration making the common domestic animals and fowls large and plump, on the statements of travelers and others as to the size and build of eunuchs, on the supposed tendency of women to become stout after the menopause, and especially on the frequency with which castration is followed by cure in cases of osteomalacia. The findings in the case of osteomalacia have indeed given rise to the view that the ovaries have an internal secretion which has some regulating action on the metabolism as a whole, and more especially on the metabolism of the bone. I have made a very careful study of the literature on the subject of castration, but, since this paper deals only with the effect of castration on the metabolism, I shall not go into the literature further than to indicate briefly along what lines investigations have been carried.

The observations made upon the size and build of animals and of domestic animals and fowls which have been castrated can, I

[1] The expenses of this research were contributed in part by the Proctor Fund for the Study of Chronic Disease, in part by the Bullard Fellowship.

think, be dismissed as not being of the nature of controlled experiments. Eunuchs are very frequently not of the same race as their masters and lead a different kind of life in many ways, so that measurements made upon them should not be compared with measurements of their masters. Regarding domestic animals and fowls, it will be sufficient to say that anyone who has compared the active quarrelsome life of a cock with that of the placid capon can see that a cock might be expected to be lean and tough.

Turning to laboratory and clinical experiences, the various experiments on the effect of feeding ovaries and ovarian extracts may be mentioned.[1] The results were all negative.

There is a very general impression among clinicians that the ovaries have something to do with the formation of blood, and that aplasia of sexual apparatus can give rise to chlorosis.[2] This view is especially prevalent among the French.[3] It was shown long ago by Virchow,[4] and again more recently by Stieda,[5] who studied many cases, that the chlorosis is in all probability not secondary to the aplasia of the genital apparatus, but that where these occur together they are both due to an antecedent developmental disturbance. The only direct experiments bearing on the subje t are those of Breuer and Seiller[6] who found

[1] A. Leowy: Ueber den Einfluss des Oophorins auf den Eiweissumsatz' *Berl. klin. Wochenschrift*, xxxvi, p. 1100, 1899. L. Thumin: Ueber den Einfluss des Oophorins auf den Eiweissumsatz des Menschen, *Therapie der Gegenwart.* N. F. ii, p. 450, 1900. M. Dalché und M. Lépinois : Opothérapie ovarienne, *Le Bulletin Medical*, xv, p. 1076, 1901. S. Neumann und B. Vas: Ueber den Einfluss der Ovarienpräparate auf den Stoffwechel *Monatschrift für Geburtshülfe und Gynäkologie*, xv, p. 433, 1902. P. Mathes: Ueber die Einwirkung des Oophorin auf den Stoffwechsel von Frauen mit und ohne Ovarien, *ibid.*, xviii, p. 261, 1903.

[2] E. Fränkel: Ueber die Combination von Chlorose mit Aplasie der weiblichen Genitalorgane, *Arch. für Gynäk.*, vii, p. 465, 1875.

[3] Etienne and Demange: La chlorose, auto-intoxication d'origine ovarienne, *La Semaine Méd.*, xviii, p. 186, 1898.

[4] R. Virchow; Ueber die Chlorose und die damit zusammenhängenden Anomalien im Gefässapparate insbesondere über Endocarditis Puerperalis, *Beitr. zur Geburtshülfe und Gynäkologie*, 1870.

[5] H. Stieda; Chlorose und Entwickelungsstörungen, *Zeitschrift für Geburtshülfe und Gynäk.*, xxxii, p. 60, 1895.

[6] R. Breuer and R. von Seiller: Ueber den Einfluss des Kastration auf den Blutbefund weiblicher Tiere, *Arch. f. exp. Path. u. Pharm.*, l, p. 169, 1903.

that castration of female dogs was followed by a decrease in the number of red cells. We can of course conclude nothing from their experiment alone.

In regard to the alleged tendency to obesity in women after castration it may be said that hundreds of cases were followed for some years by Hegar, and others[1] since his time, and these cases do not show any uniform tendency to increase in body weight. In Keppler's cases[2] of which there were fifteen which were followed for a number of years every patient grew leaner.

Many hundreds of castrated women have been followed up for months and even years, more especially by the German clinicians, in an endeavor to find if there are any mental, moral or psychical changes, or changes in the feelings in any way.[3] I have been

[1] A. Hegar: *Die Castration der Frauen vom physiologischen und chirurgischen Standpunkte aus*, Leipzig, 1878. Glaevecke: Körperliche und geistige Veränderungen im weiblichen Körper nach künstlichem Verluste der Ovarien einerseits und des Uterus anderseits, *Arch. für Gynäk.*, xxxv, p. 1, 1889. H. Liesan: Der Einfluss der Castration anf den weiblichen Organismus, Diss. Freiburg, 1896. A. Pfister: Die Wirkung der Castration auf den weiblichen Organismus, *Arch. f. Gynäk.*, lvi, p. 583, 1898.

[2] T. Keppler: Das Geschlechtsleben des Weibes nach Kastration, *Wien. med. Wochenschrift*, xli, p. 1489, 1891.

[3] A. Hegar: *Die Castration der Frauen vom physiologischen und chirurgischeu Standyuukte aus*, Leipzig, 1878. L. Ormières: Sur la menstruation après l'ovariotomie et l' hystérectomie, Thèse de Paris, 1880. H. Fehling: Zehn Castrationen. Ein Beitrag zur Frage nach dem Werthe der Castration, *Arch. f. Gynäk.* xxii, p. 441, 1884. W. Wiedow: Die Castration bei Uterusfibrom, *Arch. f. Gynäk.*, xxvi, p. 1, 1885. L. Prochownick: Beitr. zur Castrationsfrage, *Arch. f. Gynäk.* xxix, p. 183, 1887. O. Straehle: Beitr. zu den Anzeigen der Castration, Thèse, Basel, 1888. W. Wiedow: Die Definitiven Resultate der Castration bei Uterusfibrom, *Beitr. zur Geburtshülfe und Gynäk.*, 1889, p. 162. Schmalfuss: Zur Castration bei Neurosen. *Arch. f. Gynäk.* xxvi, p. 1, 183, 1885. Glaevecke: Körperliche und geistige Veränderungen im weiblichen Körper nach künstlichem Verluste der Ovarien einerseits und des Uterus andererseits, *Arch. f. Gynäk.*, xxxv, p. 1, 1889. F. Keppler: Das Geschlechtsleben des Weibes nach Kastration, *Wien. med. Wochenschr.*, xli, p. 1489, 1891. Peaslee: *Ovarial Tumors*, New York, 1872. Hermes: Ueber die Erfolge der Castration bei Myomen, *Arch. f. Gynäk.* xlviii, p. 103, 1895. H. Liesau: Der Einfluss der Castration auf den weiblichen Organismus, Diss. Freiburg, 1896. A. Pfister: Die Wirkung der Castration auf den weiblichen Organismus. *Arch. f. Gynäk.*, lvi, p. 583, 1898. P. Mundé: The Physical and Moral Effects of Absence of the Internal Female Sexual Organs, *Am. Journ. Obstetrics*, xxix, p. 289, 1899.

through all the reported cases and feel that it is safe to say that there is no general tendency to any such changes in women who have been castrated after puberty. Puech[1] has reported a case of congenital absence of the ovaries in a woman whose feelings were normal in every respect. In many cases true menstruation and molimina menstrualia persisted after castration. Manige[2] has reported a case in which castration was performed during pregnancy and followed by normal birth and milk secretion later. Hegar,[3] has also observed milk secretion after castration.

The interesting experiments on grafting and transplantation of ovaries seem to indicate that there is possibly some sort of internal secretion by the ovaries, but these experiments show effects on functions directly related to reproduction only. Knauer[4] who experimented on rabbits showed that the ovaries could be transplanted into other parts of the body where they grew, functioned, and prevented the uterus from becoming atrophied, and indeed in one case he transplanted from a normal rabbit into a castrated rabbit a piece of ovary with the result that a year later the castrated rabbit was able to become pregnant and bear young.[5] In this connection also certain experiments which have been carried out on women are of interest. Morris[6] provided a woman 20 years old who had rudimentary adnexa and infantile uterus, and who had never menstruated, with an ovarian graft from another patient with the result that the young woman menstruated in ten days. In another case of a woman with chronic metritis and septic tubal disease with almost complete obliteration, he removed the tubes and ovaries

[1] A. Puech: *Des Ovaires de leurs anomalies* iii, De l'absence des deux ovaries.

[2] T. Maniger: Die Doppelseitige ovariotomie bei Schwangeren, *Münch. med. Wochenschrift*, xlii, p. 1117, 1895.

[3] A. Hegar: *Die Castration der Frauen vom physiologischen und chirurgischen Standpunkte, aus Leipzig*, 1878.

[4] E. Knauer: Einige Versuche über Ovarientransplantation bei Kaninchen. *Centralbl. f. Gynäk.* xx, p. 524, 1896. E. Knauer: Die Ovarientransplantation, *Arch. f. Gynäk.*, lx, p. 322, 1900.

[5] E. Knauer: Zur Ovarientransplantation. Geburt am normalen Ende der Schwangerschaft nach Ovarientransplantation beim Kaninchen, *Centrabl. f. Gynäk.*, xxii, p. 201, 1898.

[6] R. Morris: The Ovarian Graft, *N. Y. Med. Journ.*, lxii, p. 436, 1895.

and transplanted a small piece of ovary. The patient became pregnant the next month. Glass[1] transplanted a healthy ovary into a woman who had been castrated two years previously. The operation was followed by a return of the catamenia. More recently Cramer[2] transplanted ovaries into a woman with amenorrhœa and small uterus. In her case the uterus increased in size, the catamenia appeared and the breasts began to swell. These experiments give us the most definite knowledge we have in regard to castration and the functions of the ovaries, although they do not give us any direct information regarding the effect of castration on the metabolism.

The effect of castration in curing many cases of osteomalaeia is well known. It has come to be believed in as a fact that this condition is a disease in which the internal secretions of the ovaries is disturbed. I shall not go into the matter here as I intend to discuss it in detail in a later paper on osteomalacia. I shall merely refer to the many cases of osteomalacia which we e not cured by castration[3] and to the paper of Bulius[4] in wh ch he criticises his earlier findings and denies that in osteomalacia there is anything peculiar n the condition of the ovaries.

I shall not discuss the results of metabolism exneriments before and after castration in osteomalacia,[5] as I intend to do

[1] J. Glass: An experiment in Transplantation of the Entire Human Ovary. *N. Y. Med. Journ.*, lxxiv, p. 523, 1889.

[2] Cramer: Ueber Transplantation menschlichen Ovarien. *Niederreinische Gesellschaft für Natur-und Heilkunde in Bonn, Deutsche Med. Wochen.*, xxxii, 1883, (1906).

[3] D. Straehle: Beitr. zu den Anzeigen der Castration, Thèse, Basel, 1888. F. von Winckel: Ueber die Erfolge des Castration bei Osteomalakie, *Volkmann's Klin. Vorträge* N. F. No. 71. E. Plagàr, Die Heilung der Osteomalacie mittelst Castration, *Arch. f. Gynäk.*, xlix, p. 30, 1895. F. McCrudden: The Effect of Castration on the Metabolism in Osteomalacia, *Amer. Journ. of Physiol.*, xiv, p. 211, 1907.

[4] G. Bulius: Osteomalacie und Eierstock, *Beitr. zu Geburtshülfe und Gynäk.*, i, p. 138, 1898.

[5] See S. Neumann: Quantitative Bestimmung des Calciums, Magnesiums und der Phosphorsäure im Harn und Koth bei Osteomalacie, *Arch. f. Gynäk*, xlvii, 1894. S. Neumann: Weitere Untersuchungen über die Stoffwechsel Verhältnisse des Calciums, Magnesiums, der Phosphor-

this in a later paper, further than to say that in this disease the metabolism of the inorganic elements is disturbed and after castration there is the effect of the disease plus the effect of castration. In most of the studies of the metabolism after castration investigators have confined themselves to a study of the phosphates in the urine. The phosphates in the feces and in most cases in the food have been neglected, so that the results are of very little value. This applies to the studies of Curatulo and Tarulli, Mossé and Oulié, Pinzani, Schulz and Falk, and Neumann and Vas.[1] Beyer[2] who analyzed the food, urine and feces of both female and male dogs before and after castration did not find that the operation has any ffect on the metabolism of phosphorus. Beyer used young dogs before the age of puberty.

According to Heymann[3] castration of rats is followed by a

säure und des Nitrogens bei puerperaler Osteomalacie, mit besonderer Rücksicht auf die durch die Castration und anderer therapeutische Eingriffe verursachten Veränderungen des Stoffwechsels, *Arch. für Gynäk.*, li, p. 130, 1896. J. Goldthwait, C. Painter, R. Osgood, and F. McCrudden; A Study of the Metabolism in Osteomalacia, *Amer. Journ. of Physiol.*, xiv, p. 389, 1905. F. McCrudden: The Effect of Castration on the Metabolism in Osteomalacia, *Amer. Journ. of Physiol.*, xvii, p. 211, 1907.

[1] G. Emilio Curàtulo und L. Tarulli: Einfluss der Abtragung der Eierstöcke auf den Stoffwechsel, *Centralb. f. Gynäk.*, xix, p. 555, 1895, also *Centralb. f. Physiol.*, ix, p. 149, 1895 and *Edinburgh Med. Journ.*, xli, p. 137, 1895. Tarulli e Curatulo: Sulla secrezione interna dell'ovaio, *Boll. d. Real. Accad. Med. di Roma*, 1896. *Maly's Jahresbericht*, xxvi, p. 559, 1896. P. Mossé et Oulié: Influence de l'ovariotomie double et de l'ingestion d'ovaires sur quelques elements de la sécrétion urinaire chez la chienne, *ibid.* 1899. Pinzani: Experimentelle Untersuchungen über den Einfluss der Kastration auf den Stoffwechsel und die Blutbeschaffenheit, *Centr. f. Gynäk.* xxiii, p. 1311, 1899. F. Schulz und O. Falk: Phosphorauscheidung nach Castration, *Zeitschr. f. physiol. Chem.*, xxvii, p. 250, 1899. O. Falk: Ein Beitrag zur Kenntniss des Stoffwechsels nach Entfernung der ovarien, *Arch. f. Gynäk.*, lviii, p. 565, 1899. S. Neumann und B. Vas: Ueber den Einfluss der Ovarienpräparate auf den Stoffwechsel, *Montaschr. f. Geburtshülfe und Gynäk.*, xv, p. 433, 1902.

[2] K. Beyer: Beitrag zur Frage des Castration und deren Folgezustände, Diss. Greifswald.

[3] T. Heymann: Zur Einwirkung der Castration auf den Phosphorgehalt des weiblichen Organismus, *Zeitschr. f. physiol. Chem.* xli, p. 246, 1904, and *Arch. f. Gynäk.*, lxxiii, p. 366, 1904.

decrease in the total amount of phosphate in the body and bones, but this is denied by both Lūthje[1] and Berger.[2]

Experiments on the metabolism of energy do not show that this is affected by castration.[3]

It is clear from the brief sketch of the literature on the subject that we have no definite knowledge concerning the effect of castration on the metabolism of normal adult animals. It was in the hope of contributing some definite knowledge that the following experiments were carried out.

THE EXPERIMENT.

In these experiments two healthy male dogs and two healthy females were used. The animals were castrated under all surgical precautions by the late Dr. Gould of the Surgical Department of the Harvard Medical School. Ether was used as anæsthetic. The wounds healed rapidly without infection. For a period of twenty days before the operation the animals were subjected to a metabolism experiment in which the calcium, magnesium, phosphorus, sulphur and nitrogen were determined quantitatively in the food, urine and feces. After the wounds had healed, another similar experiment was performed on each animal.

The metabolism cages. One of the animals was kept in a cage built according to the specifications of Dr. Gies.[4] The others were kept in a rather simpler form of cage. We were able in all cases to collect all the urine and feces separately.

The food consisted of a mixture of lean beef heart, lard, cracker dust and water in the proportions of 20 grams fairly dry ox-heart,

[1] H. Lūthjé: Ueber die Kastration und ihre Folgen, *Arch f. exp. Path. und. Pharm.*, l, p. 268, 1903.

[2] C. Berger: Beiträge zur Frage von den Folgezuständen der Kastration, insbesondere von deren Einfluss auf den Phosphor-Stoffwechsel, Diss., Greifswald 1903.

[3] A. Loewy und P. Richter; Sexual Function und Stoffwechsel, *Arch. für Physiol.*, 1899, suppl. p. 174. H. Lūthje: Ueber die Castration und ihre Folgen, *Arch. f. exp. Path. und Pharm.*, xlviii, 184, 1902. L. Zuntz: Experimentelle Untersuchungen über den Einfluss der Kastration und der Oophorindarreichung auf den Stoffwechsel der Frauen, *Zeitschr. f. Geburtshülfe und Gynäk.*, liii, p. 352, 1904.

[4] *Science*, xiii, p. 469.

5 grams fat, 3 grams cracker dust, and 40 grams water per kilo body weight per day.[1]

The food was prepared and sampled in the following manner: About 200 pounds of ox-heart were bought and the gross fat cut off and thrown away. The rest of the hearts were put through a sausage machine and divided as finely as possible. Small portions at a time were then placed in a series of small canvas bags and squeezed as dry as possible in a press. The fairly dry and minced heart meat was then thoroughly mixed on a well washed board floor. It was then sampled by quartering[2] to about 1000 grams and a sample of this weight was put aside for purposes of analysis. The remainder was again thoroughly mixed and quartered down to a number of small samples for each of the dogs. Two portions of twenty days' rations each were then carefully weighed out for each dog, each portion divided into twenty parts, each part rolled out into a ball and the whole put away in labeled pans in the cold room and kept frozen solid. The lard was melted, cooled, divided into two portions of twenty days' rations for each dog, each portion divided into twenty pieces and the whole put into the cold room.

The cracker dust and water were weighed out each day. Once a day at noon the dogs were fed. A day's portion of meat, lard, cracker dust and water were mixed together and warmed, the whole making a stew which the dogs ate with great relish.

The actual amounts fed were as follows:

	WT. IN KILOS.	MEAT PER DAY.	LARD PER DAY.	CRACKER DUST PER DAY.
		grams.	*grams.*	*grams.*
Female 1	6.2	124.0	31.0	18.6
Female 2	7.3	146.0	36.5	21.9
Male 1	8.6	172.0	43.0	25.8
Male 2	10.7	214.0	53.5	32.1

Urine. The urine from each dog was collected each day and placed in the cold room where it was frozen solid.

Feces. The feces were collected and kept in the cold room.

Analysis; Food. The thousand grams of meat saved for

[1] Recommended by Dr. Gies and found to be a fairly good average amount. The requirements of the individual dogs were found to vary in this respect however.

[2] McCrudden: Report of the 7th International Congress of Applied Chemistry. Berlin, 1903, section viii, vol. iv, p. 256.

analysis were dried, mixed and ground up, and half of it, corresponding to 500 grams meat, mixed with 75 grams cracker dust. This mixture was further dried, ground up, sampled, and weighed, and two-fifths of it, weighing 94.06 grams, and corresponding to 200 grams meat and 30 grams cracker very finely ground and put through a very fine mesh sieve. For analysis a certain amount of this mixture was mixed with a certain amount of lard, and each of the various elements determined in the mixture. For example, in the nitrogen determination 0.1881 gram of the meat and cracker powder were mixed with 0.1000 gram lard. This corresponds to 0.4000 gram of meat as fed to the dog; 0.0600 gram cracker, and 0.1000 gram lard; and this mixture is equivalent to one-fiftieth of a day's rations per kilo dog. The quantity of nitrogen in the food of the dog for the whole period would be the amount in this sample multiplied by the factor 50, this by the number kilos the dog weighed, and this by 20, the number of days of the experiment.

Urine. The urine for each dog for the whole twenty days was mixed together, measured, and an aliquot part taken for analysis.

Feces. The feces were finely ground and an aliquot part taken for each analysis.

Analytic Melhods. The methods of anaylsis were those used in previous metabolism experiments.[1]

Data.

Weight of the Dogs: The health of the dogs remained good throughout. Their weights were as follows:

	FEMALE 1.	FEMALE 2.	MALE 1.	MALE 2.
Before first experiment........	13.7	16.0	19.0	23.5
After first experiment.........	13.7	16.5	19.0	23.0
Before second experiment.....	13.2	16.0	19.0	22.0
After second experiment.......	13.4	16.4	18.2	21.2

The amounts of urine[2] were as follows:

[1] *Amer. Journ. Physiol.*, xiv, p. 389.

[2] The amount was a trifle less than this in most cases, and was made up to the nearest hundred centimeter with distilled water to simplify the process of taking an aliquot part.

	FEMALE 1.	FEMALE 2.	MALE 1.	MALE 2.
	cc.	*cc.*	*cc.*	*cc.*
Before castration, first experiment	2175	6730	7000	10300
After castration, second experiment	4500	6000	6300	4500

The quantity of dried feces was as follows:

	FEMALE 1.	FEMALE 2.	MALE 1.	MALE 2.
	grams.	*grams.*	*grams.*	*grams.*
Before castration	101.5	135.9	111.3	89.5
After castration	93.0	116.3	173.0	170.2

The results of the metabolism experiments were as follows:

Metabolism of 18 lb. Female before Castration.

	NITROGEN.	SULPHUR.	P_2O_5.	CaO.	MgO.
Urine	46.55	2.484	7.197	0.0533	0.2236
Feces	10.29	1.126	2.168	0.476	0.846
Total excretion	56.84	3.610	9.365	0.529	1.070
In food	108.3	6.59	15.90	0.508	1.461
	+51.5	+2.98	+6.54	−0.021	+0.391

After Castration.

	NITROGEN.	SULPHUR.	P_2O_5	CaO.	MgO.
Urine	92.8	5.22	12.68	0.074	.303
Feces	11.31	1.351	3.239	.458	.768
Total excretion	104.1	6.57	15.92	.532	1.071
In food	108.3	6.59	15.90	.508	1.461
	+4.2	+0.02	−0.02	−0.024	+0.390

Metabolism of 16 lb. Female before Castration.

	NITROGEN	SULPHUR.	P_2O_5	CaO.	MgO.
Urine	112.7	6.874	16.30	0.215	0.718
Feces	12.91	1.538	6.642	6.53	0.928
Total excretion	125.6	8.412	22.94	6.745	1.646
In food	127.5	7.76	18.72	.599	1.720
	+1.9	−0.65	−4.22	−6.146	+0.074

After Castration.

	NITROGEN.	SULPHUR.	P_2O_5.	CaO.	MgO.
Urine	107.9	6.21	14.57	0.084	.371
Feces	12.04	1.466	3.953	1.785	1.279
Total excretion	119.9	7.68	18.52	1.869	1.650
In food	127.5	7.76	18.72	.599	1.720
	+7.6	+0.08	+0.20	−1.270	+0.070

Metabolism of 19 lb. Male before Castration.

	NITROGEN.	SULPHUR.	P_2O_5.	CaO.	MgO.
Urine	126.9	7.331	17.89	0.172	0.506
Feces	10.89	1.499	2.263	0.768	0.595
Total excretion	137.8	8.830	20.15	0.940	1.101
In food	150.2	9.14	22.05	.705	2.026
	+12.4	+0.31	+1.90	−0.235	+0.925

After Castration.

	NITROGEN.	SULPHUR.	P_2O_5.	CaO.	MgO.
Urine	130.6	7.43	18.27	.198	0.4284
Feces	21.88	2.656	5.318	2.01	2.011
Total excretion	152.5	10.09	23.59	2.208	2.439
In food	150.2	9.14	22.05	0.705	2.026
P	−2.3	−0.95	−1.54	−1.503	−0.413

Metabolism of 23 lb. Male before Castration.

	NITROGEN.	SULPHUR.	P_2O_5.	CaO.	MgO.
Urine	161.9	9.727	23.51	0.350	0.819
Feces	6.72	1.186	2.99	2.76	2.41
Total excretion	168.6	10.913	26.50	3.11	3.23
In food	186.9	11.37	27.43	.877	2.520
	+18.3	+0.46	+0.93	−2.233	−0.710

After Castration.

	NITROGEN.	SULPHUR.	P_2O_5.	CaO.	MgO.
Urine	169.8	9.32	22.66	0.313	0.748
Feces	19.80	2.746	5.92	4.12	1.587
Total excretion	189.6	12.07	28.58	4.433	2.335
In food	186.9	11.37	27.43	.877	2.520
	−2.7	−0.70	−1.15	−3.556	+0.185

Discussion of the Results. The results are such that they do not require a detailed discussion. It will be seen that they do not confirm the view generally held that castration is followed by a general retention of material, especially the mineral elements of the body. In fact, my results show a general tendency in the

other direction. Where there was a retention of material before castration this retention was either decreased or became a loss after castration in almost every instance.

I think we can conclude that castration does not cause a decrease in oxidation processes and a retention of material. The work was undertaken primarily to see what light it might throw on the view that osteomalacia is a disease of the ovaries. I shall be able to use the results obtained as an argument against this view.

CHEMICAL ANALYSIS OF BONE FROM A CASE OF HUMAN ADOLESCENT OSTEOMALACIA.

By FRANCIS H. McCRUDDEN.

(From the Laboratory of Biological Chemistry of the Harvard Medical School.)

(Received for publication January 5, 1910.)

In a previous paper[1] I gave the results of analysis of bone from a case of osteomalacia in a horse, and compared the results with the normal. It was seen that in osteomalacia the calcium was low and the magnesium and sulphur high, a result in accord with metabolism experiments in this disease. The results lend support to Cohnheim's view based on anatomical evidence, that this disease is not a condition of simple solution of the inorganic constituents, but that wherever bone is taken up both inorganic and organic constituents as a whole are taken up and that new bone tissue laid down is osteoid tissue poor in lime.

I recently had the opportunity to analyze a piece of bone from a case of human osteomalacia through the k ndness of Professor Painter of Tufts Medical School. It was examined microscopically by Prof. Leary of Tufts Medical School and pronounced to be a specimen of adolescent osteomalacia.

A sample of normal human bone was analyzed for comparison. The methods of analysis were those used in the previous analyses.

The results were as follows:

	OSTEOMALACIA. *Per cent.*	NORMAL *Per cent.*
CaO	15.44	28.85
MgO	0.57	0.14
P_2O_5	12.01	19.55
S	0.55	0.14

[1] F. McCrudden: The Composition of Bone in Osteomalacia, *Amer. Journ. of Physiol.*, xvii, p. 23, 1907.

The results here are similar to those obtained in the case of the horse, and are in accord with Cohnheim's theory and not with the theory of Virchow that the process is one of simple halisteresis. The quantity of magnesium and sulphur is increased out of all proportions to decrease in the quantity of calcium, a condition which corresponds not with a fixed content of magnesium and sulphur, which increases proportionally as the calcium decreases, but with an absolute increase in the amount of sulphur and magnesium. And this is what we should expect with a laying down of new tissue rich in magnesium and sulphur.

CORRECTION OF AN OMISSION

IN THE PAPER ON "THE QUANTITATIVE SEPARATION OF CALCIUM AND MAGNESIUM IN THE PRESENCE OF PHOSPHATES AND SMALL AMOUNTS OF IRON DEVISED ESPECIALLY FOR ANALYSIS OF FOODS, URINE AND FECES."[1]

By FRANCIS H. McCRUDDEN.

Through an oversight a very important step in the determination of calcium is omitted in the final description of the methods,—namely, the addition of sodium acetate after the addition of ammonium oxalate,—without which the method is of no value. After the second paragraph on page 99, the following paragraph should be inserted:

"After the precipitation has become crystalline, and has settled, the solution is allowed to cool. It should not be above the room temperature. When cold, add to it slowly and with constant stirring, 8 cc. of the 20 per cent sodium acetate solution."

[1]This *Journal*, vii, p. 83, 1910.

THE INFLUENCE OF DIETARY ALTERNATIONS ON THE TYPES OF INTESTINAL FLORA.

(Plates I–III.)

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AND

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(Received for publication, December 24, 1909.)

The first systematic investigation of the fecal flora was that made by Escherich[1]. This observer, using modern aërobic and anaërobic cultural methods, isolated from the dejecta of normal nurslings and bottle-fed babies some of the best known of the intestinal bacteria. Subsequent workers have enriched the literature of intestinal bacteriology with scores of more or less imperfectly described and incompletely identified organisms, without, however, studying the newly-isolated bacteria either with respect to their numerical relations to other fecal bacteria or from the standpoint of their chemical activities within the intestine.

The discovery of the etiological relationships of the typhoid, dysentery and cholera organisms to these diseases also diverted attention from the normal intestinal bacteria, and at once directed the efforts of investigators toward the isolation of bacterial "species" which might be the causative agents of a variety of intestinal ailments.

In a previous communication[2] attention was directed to the chemical inactivity, or, more correctly, the inability of many exogenous pathogenic bacteria to produce deep-seated changes in artificial media. It would appear, inasmuch as the more promi-

[1] *Die Darmbakterien des Säuglings*, Stuttgart, 1886.

[2] Kendall, A. I.: This *Journal*, vi, p. 499, 1909.

nent normal bacteria are able to initiate marked changes in their environment, that there is a fundamental difference between these two types of bacteria. It is thus not difficult to see, that the lack of appreciation of this important difference is an element in causing the present unsatisfactory state of our knowledge of the intestinal bacteria.

The present studies are based upon an entirely different intent from that of previous investigators. Instead of *isolating* a series of cultures from the dejecta of man and animals, and studying them in the greatest detail, an attempt has been made to demonstrate definite relations between the nature of the diet on the one hand, and the leading characteristics of the resulting bacteria on the other, including some products of their vital activity.

There are many clinical indications in man that sharp alternations in the chemical nature of the diet are attended by rapid changes in the physiological state of the digestive tract. For example it is well known to physicians that the free use of carbohydrates (e. g., lactose) in bottle-fed infants is commonly followed by a softening in the consistence of the feces and perhaps by the signs of excessive fermentation. The return to a milk diet restricted in carbohydrates is followed by a prompt recession of these signs of increased fermentation. Similarly it is a familiar observation that certain undesirable symptoms (lassitude, drowsiness, headache) attendant on the use of an excessive share of protein in the diet are relieved by a restriction of proteins and a freer use of carbohydrates. Although it has sometimes been assumed that alterations in the bacterial flora attend such changes in diet, we know of no studies designed to determine the precise effects on the nature of the flora that may arise from definite and abrupt variations in the chemical composition of food. In our experiments we have asked ourselves if it is possible to establish quite definite unchallengeable concomitant variations in food and bacteria, since the establishment of such variations, under physiological conditions, must be an essential condition for the discovery of exact indications for diet under states of infection in the intestine. In order to secure conditions as favorable as possible to clarity and definition of results we have planned our experiments so as to obtain somewhat extreme

physiological dietaries. Thus we have alternated a diet consisting mainly of protein with a diet in which carbohydrates have been more than ordinarily prominent and vice versa. We have furthermore made the alternations abrupt in order to leave the least possible chance for gradual adaptation to a given class of dietary.

These experiments were carried out upon two widely different types of mammals—monkeys and cats. The difference in the types of experimental animals selected served to test the question whether the same alternations in diet would yield the same results in bacterial change in omnivora and carnivora. The advantage of using monkeys as experimental animals, similar to man in their digestive processes, is obvious.

The diets selected consisted of meat for the cats and eggs for the monkeys as representative of protein foods. Milk to which was added dextrose was selected as the carbohydrate diet, experience having shown that this milk and sugar combination exhibits, from a bacterial point of view, the properties of carbohydrate food.

At first sight it would appear that eggs are an unusual diet for monkeys and that the results might be different from those which would obtain in normal primates, but it should be remembered that bird's eggs are by no means an uncommon delicacy in the diet of normal monkeys. Milk forms an essential part of the pabulum of these animals in their early life. Both meat and milk are common articles of food in the dietary of cats and need no further comment. As will be shown later, the striking fact brought out by these experiments is that the same bacterial changes occur with the same types of diet in the different types of animals irrespective of the apparent novelty of the food. And because of this observed regularity of relation between the nature of the diet and the character of the intestinal flora the results are all the more noteworthy.

The methods used in this investigation, described in a previous communication[1] differ from those previously described, not in their nature, but in the manner of utilizing them. Cultural media of appropriate composition have been used as specific

[1] Kendall, A. I.: *loc. cit.*

enrichment media in such a manner as to indicate the relative distribution of the acidophilic and protein flora as the diet is changed from carbohydrate to protein and vice versa.

The use of fermentation tubes containing dextrose, lactose and saccharose broth, as well as milk fermentation tubes has been particularly helpful, and the study of the Gram-stained sediments from these tubes, particularly the broth tubes, has furnished clues which have led, through the mediation of plating, to the emergence of valuable data.

It should be stated that the fecal flora are less responsive, or, better, respond less completely in the earlier changes in diet, although even in the first changes in diet the variations are strongly marked. As the experiments are continued, there is a tendency toward a unification of the flora, due apparently to the gradual suppression of those organisms which are less easily habituated to the protein or carbohydrate regimen. While at first sight this might seem to be a source of error, further consideration shows that this very fact is in itself strong presumptive evidence of the correctness of the results obtained. As the diet is changed from the protein to the carbohydrate, and back again the flora exhibit a stronger and stronger tendency to become simplified, until finally the protein bacteria and the acidophilic bacteria crowd out the more or less ubiquitous organisms and the flora eventually respond with precision and promptitude to the changed dietary conditions.

The general plan followed was to place the animals upon a protein diet for one or two weeks, then to shift abruptly to the carbohydrate regimen for the same length of time, reversing the diets at regular intervals as indicated above. These intervals were found to be sufficiently separated to permit of the full development of the respective flora. At the same time they were found to be sufficiently far apart to prevent confusion of the two types of bacterial response to the dietary changes, such as is liable to occur when the alternations of diet are made too frequently. In the latter event the bacterial response reflects in part the protein regimen, in part the carbohydrate, and the results cease to be clear cut.

PHYSIOLOGICAL EFFECTS OF DIET.

As the proteolytic bacteria become dominant in the alimentary canal, the monkey becomes sleepy and rests on its perch with its head bowed in its hands; it is stupid and responds slowly to external stimuli, takes its food very deliberately, and manifests little interest in its surroundings. Not infrequently the animal even after a hearty meal will spend much time trying to bite the woodwork of its cage.

The urine is voided in small amounts daily and is relatively highly colored. The amount may, roughly, be considered to be one-half that which is obtained from a carbohydrate diet such as employed. In the urine as the proteolytic bacteria become fully established there is an abundance of indican as well as aromatic oxyacids. The urorosein reaction due to indolacetic acid is rarely observed and at best very faintly. The lower specific gravity of the urine during the milk periods has to be taken into account in estimating the intensity of the reactions for indican and aromatic oxyacids. No observed differences in the concentration of the urines of the different periods can account for the variations in these reactions which were actually observed.

The fecal mass is small, rather desiccated and distinctly yellow in color or yellowish-brown. The odor is strongly suggestive of indol or skatol.

As the diet is changed to carbohydrate (best done by feeding the animal milk containing a moderate amount of dextrose), both the psychical and the physical attitude of the animal undergo a great change. The monkey no longer holds its head in its hands, the posture is erect, the animal is alert and bright, notices everything, reacts promptly to all kinds of stimuli and eats abundantly. The eyes lose their dull, lustreless appearance and become bright. The animal no longer attempts to chew the woodwork of its cage.

The urine becomes more voluminous, approximately twice its previous volume and pale in color. The indican and aromatic oxyacids grow much less or disappear. Any traces of indolacetic acid also disappear.

The feces, at first diarrhœal become formed, gray in color, and

inoffensive in odor, contrasting markedly in this respect with the feces derived from a protein diet.

BACTERIOLOGICAL CONDITIONS.

Morphology. As the "protein flora" becomes established the Gram-stained fields show considerable numbers of large, Gram-positive bacilli with rounded ends, which rarely or never occur in chains but not infrequently occur in pairs. Smaller Gram-positive organisms also with rounded ends, but showing a distinct tendency to become spindle-shaped particularly as sporulation approaches, are also seen. The latter, and to some extent the former, may under certain conditions, particularly when there is a change of diet, become Gram-negative and show undoubted signs of degeneration. This degeneration is usually accompanied by vacuolation. Besides these Gram-positive organisms, Gram-negative organisms referable, morphologically, to *B. coli*, are abundant. Other organisms from time to time make their appearance; these last mentioned organisms, however, are inconstant, and apparently are to be regarded as of secondary importance.

Cultural Features. The mixed fecal flora, when inoculated into dextrose, lactose and saccharose fermentation media, produce typically considerable volumes of gas, not infrequently amounting to 90 or even 100 per cent of the length of the closed arm of the fermentation tube. The fermentation medium becomes very turbid, and an abundant sediment collects at the foot of the closed arm. This sediment, stained by Gram's method, shows the same types of organisms that were present in the Gram-stained smears prepared from the feces direct. Fermentation tubes containing milk also show an abundance of gas. The milk is very considerably peptonized and usually the undissolved coagulum is colored brown. As a rule no distinct odor of butyric acid is detectable in either the carbohydrate fermentation tubes or the milk fermentation tubes. Gelatin stab cultures inoculated as before with the mixed fecal flora, show somewhat rapid peptonization. This peptonization usually assumes the form of a rather deep funnel, indicating that the bacteria bringing about the change are not obligate anaërobes. The acid dextrose broth shows few bacteria of the acidophilic type.

The transitional flora, as the animal changes from protein to carbohydrate is characterized by two noteworthy features; first the bacteria become much smaller and stain rather poorly. Evidences of degeneration are seen, particularly vacuolation, in certain instances spore-formation has been a feature as well. Secondly, great irregularities in the distribution of the various types occur. These irregularities, which are not only morphological but are found to be cultural as well, are apparently due to the antagonism which occurs between the protein and the carbohydrate flora. As the latter gradually becomes dominant, the Gram-stained fields which, on the protein diet were heterogeneous in appearance, tend to become more homogeneous and the most prominent organisms are Gram-positive rods, thinner than those noted in the protein diet, and somewhat longer. These rods, indeed, are so abundant that the fields resemble in a striking manner those of normal nurslings. Cultural investigation has shown that these organisms are in reality closely allied to those characteristic of the normal nursling flora. It is only by careful scrutiny that a few residual organisms of the protein diet, particularly those resembling the smaller, Gram-positive organisms described above, can be found.

Culturally the conditions are exactly the reverse of those obtaining on the protein diet. In the fermentation tubes the gas volume rapidly decreases and ultimately practically disappears. The turbidity becomes very much less marked and, indeed, in some cases only a faint opalescence develops. The sediments are composed almost wholly of rather elongated, Gram-positive rods, agreeing with those found in the feces. In addition, under certain not well understood conditions, rod-shaped organisms with bifid ends are seen. These organisms in reality are to be regarded as *B. bifidus*. In several instances it has been possible to isolate these bifid-like organisms and in their cultural complex and general morphology they are to be regarded as identical with Tissier's organism.

The milk fermentation tubes show a very slight growth, and in fact, the only visible indication of bacterial activity is a slight coagulation. The organisms of the carbohydrate flora do not cause pronounced peptonization or free gas-formation in this medium. In gelatin the growth is very scanty and only after

several days is it usual to find signs of bacterial development. The organisms which occur are to be regarded as proteolytic bacteria which have developed in this medium.

Acidophilic bacteria do not ordinarily grow in gelatin and the value of the gelatin tubes in this connection is principally that they furnish an environment so favorable to the proteolytic flora and so unfavorable to the carbohydrate flora that the former, even when present in very small numbers, and with greatly diminished vitality, can grow. In the acid dextrose broth the acidophilic bacteria find a very favorable medium and even in the highest acidities they grow readily, contrasting in this respect with the lack of growth in the medium which is so characteristic of the protein flora.

Much attention was given to the question of the presence of strict anaërobes, especially the gas bacillus (*B. perfringens, B. aërogenes capsulatus*) as it is now well known that organisms of this type are liable to be present in the intestine of many animals. Many attempts were made to isolate the gas bacillus from the feces of the experimental monkeys but without success. In one instance (Monkey No. III) the animal was killed after having been fed on a protein diet and cultures were made from various levels of the intestinal tract. Material obtained from various levels in the large and small intestine was suspended in sterile salt solution and injected into the ear veins of rabbits. The animals were killed after a short interval and incubated at 30° for eighteen hours. The livers were found to be dark colored and to have a putrefactive odor but not the odor of butyric acid. There was no development of gas in the livers. The microscopical appearances obtained in smears made from the liver were like those obtained from the liver of an animal subjected to the Welch-Nuttall procedure after the intravenous injection of a culture of the gas bacillus. The bacteria were very abundant and had the morphological appearance of the gas bacilli. The appearances indicated that they were present in almost pure culture. Subsequent cultural study, however, made it clear that these organisms could not be classed with the gas bacilli. Inoculated into milk fermentation tubes they failed to induce the characteristic stormy fermentation with butyric acid production which one expects to find where one is dealing with the gas

bacillus. Moreover our organisms grew aērobically on dextrose-agar plates. They liquefied gelatin. We are strongly disposed to class these organisms observed by us as members of the *B. subtilis* group.

Pure cultures of the organisms which we have just described were injected into the leg muscles of guinea-pigs. They gave rise to a moderate hemorrhagic œdema at the site of inoculation. The bacilli were recovered in pure culture from the site of lesion and again inoculated into rabbits by the Welch-Nuttall method. It was again found that they had multiplied very abundantly under anaērobic conditions in the liver and presented an appearance like that observed where the gas bacillus is present. Again they failed to induce gas production in the liver.

While these experiments do not absolutely exclude the presence of the gas bacillus in the intestinal contents of our monkeys, they render it highly probable that this organism was not an important feature in the intestinal contents of the monkeys fed on a protein diet. In the case of the kittens no careful study was made to determine the presence of the gas bacillus. It is not unlikely from what we know of the intestinal bacteria in cats on a meat diet that this organism was present. Our experience in the case of the monkeys showed us, however, that the presence of the gas bacillus is not essential to an explanation of the phenomena which we have described in this study.

The tables are for the most part self-explanatory. The gas produced in the dextrose, lactose and saccharose tubes is expressed in percentages of the total length of the tube, instead of millimeters, experience having shown that the former method is more accurate. The readings were made at the end of eighteen hours incubation at 37°.

The term "type" occurs in two different places; the first in connection with the fermentation tube sediments, and directly stained smears, in which the +, − and ± mean, respectively, positive staining organisms (gram stain), negative staining organisms, and (in the ± fields) an approximately equal division between the positive and negative types. In other words, the + and − indicate the dominance of Gram-positive and Gram-negative bacteria respectively. In the fermentation tubes, associated with *gas*, the term is used to indicate the amount of

growth as indicated by the turbidity in the closed arm of the fermentation tube.

The action of the bacteria on milk is recorded in percentages of gas formed and in the occurrence of coagulation and peptonization in the milk fermentation tubes.

The indican was tested for in the usual manner, utilizing the Obermeyer reagent for the purpose, and shaking out with chloroform. Aromatic oxyacids were sought by the use of Millon's reagent. Sodium nitrite was used in seeking indolacetic acid.

The acidophiles were grown in media having an acidity of $\frac{N}{20}$ $\frac{N}{10}$ and $\frac{N}{5}$ acetic acid respectively. The intensity of growth is indicated by the use of the + signs, in the same manner as the relative distribution of the bacteria, above noted. Slight growth is shown by vertical lines.

It should be definitely stated that the inferences expressed in the tables as to bacterial types in the Gram-stained fields and in the fermentation tube sediments were based on extended experience with the isolation and identification of these forms by plate culture and other usual bacteriological methods. Such culture methods could not, of course, be carried out on each day's material but they were employed often enough to enable us confidently to correlate these more elaborate findings with the microscopical appearances in the fecal and sedimentary fields.

It seems worth while to comment briefly on some features of our feeding experiments which are recorded in the tables.

Table I relates to Kitten A, kept at first on a milk diet from which a sudden transition was made to a meat diet. The Gram-stains underwent changes corresponding to this pronounced alteration in diet. The acidophilic bacteria were diminished by the change from milk to meat and a similar change was observed in the fermentation tube sediments. On the other hand the subtiloid types of organisms were markedly increased by this change, especially the large subtiloid forms. Organisms of the *B. coli* group were also definitely increased by this change, the appearances in the fecal smears being confirmed by those obtained in the fermentation tube sediments. A slight increase in coccal forms was observed.

Another and very striking feature is the change from low gas production in the fermentation tubes during the milk period to high gas production during the meat period. The difference relates almost equally to the dextrose, lactose and saccharose tubes. In the urine an increase was observed in the indican and in the aromatic oxy-acids obtained with Millon's reagent.

In Table II (Kitten B), the reverse experiment was made from that recorded in Table I, namely, a change was made from meat to a milk diet. This change was associated with a fall in the numbers of large and small subtiloid organisms and a rise in the acidophilic types. There was also a diminution in the organisms of the *B. coli* type, both in the direct smears and in the fermentation tube sediments. In the fermentation tube sediments there was also a fall in the number of coccal forms observed. The gas production in the fermentation tubes was high during the meat period. During the milk period the gas production gradually fell so that toward the end of the period there was again a striking difference observable in the percentage of gas formed in the different tubes. The urine showed a fall in indican and in the reaction for aromatic oxy-acids obtained with Millon's reagent. The action of the mixed bacteria on milk is also worthy of note. Coagulation was more pronounced in the tubes made from the meat period than in those prepared from the milk period. There is also a tendency to a fall in the gas production in milk. No difference in peptonization of the coagulum was observed.

Table III (Kitten C), records an experiment similar to that given in Table II, the change of diet being from meat to milk. The features to be noticed in this table are the rise in the number of acidophilic forms, both in the direct stains and in the stains with the fermentation tube sediments; following the change to a milk diet; the diminution in the number of subtiloid organisms, large and small, both in the feces and in the fermentation tube sediments; the fall in the numbers of the colon bacilli and in the numbers of coccal forms. A fall in the percentages of gas produced in the dextrose, lactose and saccharose tubes is also a noteworthy feature, the contrast between the two periods being extreme. As in the other experiments, there was a decline in the indican and in the aromatic oxy-acids observable with Millon's reagent, in this case both these reactions becoming suppressed rather abruptly with the inauguration of the milk diet. The coagulation of milk was less marked in the milk period than in the meat period, and there was also a fall in gas production in milk comparable with that which was observed in Kitten B.

The observations made upon monkeys are included in Tables IV, V, VI and VII.

In Table IV are given observations on the control monkey on a mixed diet including bread and bananas. The animal showed considerable irregularities in regard to some of the organisms observed. Thus there was an increase after the middle of the experiment in the large subtiloid organisms. There were also irregularities in the numbers of colon bacilli observed in the fermentation tube sediments and in the number of cocci. Furthermore there were considerable irregularities with regard to the gas production in the various fermentation tubes. During the early part of the experiment the irregularities were less than those observed toward the end, when the gas volume was, for some unknown reason, quite small on some days and much larger on others. Considerable irregularities were also noticed in the gas production in milk.

In Table V (Monkey II) is presented an experiment in which the diet was changed from bread and bananas to milk, then to eggs, then back to milk, then to eggs and back again to milk. It is not necessary to comment in detail on this table. It shows essentially the same variations as those observed in the kittens when the diet was shifted from one of milk to one of eggs; or from eggs to milk. The same phenomena as were observed in the case of the kittens, when the change was made from meat to milk or vice versa, are observed here, namely, variations of the acidophiles, in the organisms of the subtiloid type, and variations in gas production. Very striking is the abrupt rise in the gas production under the influence of eggs and its subsequent fall under the influence of milk and sugar. The records as regards the putrefactive substances in the urine are incomplete but point to a decline in such products during the milk period.

Table VI (Monkey III) like the preceding one, records the results of successive alternations in diet from milk to eggs. Essentially the same comments as apply to Table V (Monkey II) find place here. Again the effect of the diet on the acidophiles and subtiloid organisms and the gas production is very marked. Although there are some irregularities in the gas production, the figures of the table indicate in a convincing way the influence of the protein diet in causing a rise in the formation of gas and the contrary influence of the milk and sugar diet. A similar influence is seen in the rise of gas production on a milk medium under the influence of the egg diet.

Table VII (Monkey IV) records another experiment illustrating the effect of change from a milk to an egg diet. In this experiment the acidophilic bacteria did not appear in connection with the milk diet, and it is also noteworthy that the subtiloid organisms do not appear to have been as much affected by the changes in diet as in previous experiments, although the fermentation tube sediments showed an increase of the large subtiloid forms during the egg diet. The organisms of the *B. coli* group also appear to have been less affected than usual by the dietary changes. On the other hand the gas production in the fermentation tubes shows in a striking way the typical effects of the change from milk to egg diet. The gas production was very low throughout the milk diet and promptly rose to a high level and maintained this high level throughout the egg period. Toward the end of the egg period there was a rise in the indican. The influence of the bacteria from the milk period and the egg period respectively on milk medium was less in this experiment than in the others that have been recorded.

One of the most notable features of our investigation is the contrast in the gas production correlated with the changes in diet. We deem it desirable to say that although we have given this phenomenon considerable attention we are still without an explanation which is satisfactory. At first it seemed possible

that the high gas values for protein diet were due to the activity of the gas bacillus (*B. aërogenes capsulatus*) but, as already stated, we were unable to prove the presence of this organism in the intestine of monkeys on protein diet. Experimental combinations of the prominent subtiloid organisms, already mentioned, with colon bacilli failed to give gas volumes approximating those which are recorded in our table. The possibility of the presence of the abundant gas formers, *B. cloacæ*, has not been fully excluded.

In addition to the studies on kittens and monkeys some observations were made by one of us (H) on a human subject in good health, with a view to determining the influence of the addition of from 100 to to 200 grams of cane sugar daily to the ordinary mixed diet containing meat. Under this addition of sugar the feces became soft and acid in reaction and odor, and the indol, skatol and phenol were diminished markedly. The acidophilic types of bacteria were distinctly increased. The influence (if any) exerted on other kinds of bacteria was not studied. The gas production by the mixed fecal flora, grown on saccharose, lactose and dextrose bouillon was markedly diminished, but the depression was from a lower level than in the case of the experiments on kittens and monkeys.

One of us (H) has made the following observations on the influence of alterations in diet on the composition of the intestinal contents with regard to putrefactive products, especially indol, skatol and hydrogen sulphide.

In a monkey receiving two eggs daily there was a rise in the amount of indol and in the amount of skatol detectable. The reactions after reaching a maximum several days after the use of this diet, continued strong during the remainder of the ten day period on this diet. On changing the diet to one consisting of 750 cc. of milk and 10 grams of dextrose daily, there was a prompt fall in the intensity of the indol and skatol reactions obtainable from the intestinal contents. After four days the reactions of these substances were only slight; after seven days, only the faintest traces were observed under conditions comparable to the preceding test.

The following values were obtained for the hydrogen sulphide bound to the feces under varying conditions of diet:

		FECES.		HYDROGEN SULPHIDE.
		gms.		gm.
On an egg diet		3.3	yielding	0.1166
" " " "		7.5	"	0.0488
On a milk-dextrose diet, after	3 days	8.	"	0.0097
" " " " "	7 "	23.	"	0.0056
" " " " "	10 "	10.	"	0.0007
" " " " "	14 "	19.	"	0.0043

SUMMARY.

Our experiments on kittens and monkeys show that an abrupt change in diet from a dominantly protein (meat and eggs) to a milk and sugar diet is followed by an alteration of physiological conditions in three distinct directions (a) in the nature of the intestinal flora; (b) in the putrefactive products of the feces and urine; and (c) in the clinical conditions.

The chief characteristic of the bacterial change is the gradual but rapid substitution of an acidophilic non-proteolyzing type of flora for a strongly proteolyzing type. The chief feature of the putrefactive conditions in the intestine is the reduction of the indol, skatol, phenol and bound hydrogen sulphide and a diminution in the indican and aromatic oxyacids of the urine. Clinically the most striking feature of the change in diet is (in monkeys) an improvement in spirits and activity which may safely be construed as showing a markedly improved sense of bodily and psychical well being.

It seems fair to ascribe these very definite changes of melioration in part to a somewhat reduced intake in protein material, since in the change from a meat or egg diet to a milk and sugar diet, there has generally been some diminution in protein. It is well known that other conditions remaining unchanged, a diminution in protein intake is followed by diminished intestinal putrefaction. It is our opinion, however, that the mere reduction in protein in our experiments is not adequate to explain the changes noted. Nor is it likely that the change in the chemical nature of the proteins ingested, as from the protein of beef or eggs to the protein of milk, is a prominent influence in effecting this change. It seems much more probable that changes in the flora observed by us are due mainly to the influence of the car-

bohydrates and that the diminished intestinal putrefaction is due to the combined influence of a diminution in protein and the addition of carbohydrate materials to the diet.

One of the most interesting features of our studies is the extensive bacterial degeneration which follows the change from one diet to another. It is possible that in conditions of disease of the intestinal tract where undesirable bacteria abound both on a protein diet and on a diet rich in carbohydrates, frequent alternations in the chemical nature of the diet are beneficial by interfering with the establishment of any one type of bacteria in the intestine.

The physiological alternations in the flora which we may claim to have established by our studies have been noted by us only in normal animals. We have as yet made no effort to study the effects of diet on animals in which the digestive tract is the seat of inflammation. It is our intention to determine what influence, if any, is exerted by pathological processes on the laws of bacterial alternation which we have noted in health.

We are unable to state whether the processes which we have studied in animals hold sway in man but consider it likely that analogous conditions will be found to exist.

TABLE I.

Kitten A.

DATE.	FECES. GRAM STAIN.											FERMENTATION TUBE SEDIMENTS GRAM STAIN.										
	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.
VII—13	−	+	−	−	−	?	−	−	−	+	+	++	+	−	−	−	−	−	−	−	+	+
14	−	+	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	+	+
17	−	+	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	+	+
18	−	+	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	+	+
19	−	+	−	−	−	−	−	−	−	+	+	−	++	−	−	−	−	−	−	−	+	+
21	−	+	−	−	−	−	−	−	−	+	+	−	+	+	+	−	+	+	−	−	+	+
24	−	−	+	+	−	+	−	−	−	+	±	−	−	+	+	−	+	−	−	−	+	±
26	−	−	++	+	+	+	+	−	−	+	±	−	−	++	+	+	+	−	−	−	+	±
30	−	−	++	+	−	++	+	−	−	+	±	−	−	++	+	+	+	−	−	−	+	±
VIII—2	−	−	++	+	+	++	+	−	−	+	±	−	?	++	+	+	++	−	−	−	+	±
3	−	−	++	+	+	++	+	−	−	+	±	−	?	++	+	−	++	−	−	−	+	±
5	−	−	++	+	−	++	−	−	−	+	±	−	−	++	+	−	++	−	−	−	+	±
7	−	−	++	+	+	++	−	−	−	+	±	−	−	++	+	−	++	−	−	−	+	±
9	−	−	++	+	−	++	+	−	−	+	±	−	−	++	+	−	++	+	−	−	+	±

TABLE I—Continued

Fermentation tubes.				Urine.			Milk.			Diet.	Remarks.
Dextrose.	Lactose.	Saccharose.	Type of growth.	Indican.	Indolacetic acid.	Millon's.	Coagulated.	Gas.	Peptonized.		
0	0	1	+							Milk and dextrose.	
0	0	0	+							"	
0	0	1	+	−	−	−				"	
0	0	4	+							"	
5	2	5	+	−	−	−				"	Meat begun.
0	8	8	+							Meat	VII—22
75	70	90	++	++	−	+++				"	
55	65	75	++	++	−	+++	+++	100	+	"	
85	65	85	++	+	−	+++	+++	100	+	"	
50	70	95	++			−				"	
80	75	80	++							"	
70	90	100	++	+	−	+++				"	
55	55	90	++							"	
90	85	95	++							"	

TABLE II.

Kitten B.

Date.	Feces. Gram stain.											Fermentation tube sediments. Gram stain.										
	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.
VII—18..	–	–	+	+	–	+	–	–	–	–	±	–	?	+	–	–	+	+	–	–	–	±
22..	–	–	++	+	++	+	–	–	–	–	±	–	?	++	+	+	+	–	–	–	+	±
23..	–	?	++	+	–	+	+	–	–	–	±	–	–	++	+	–	++	–	+	–	+	∓
25..	?	–	++	+	+	+	–	–	–	+	±	–	–	+++	+	–	+	–	+	–	+	∓
26	–	–	++	+	+	+	–	–	–	+	±	–	–	+++	+	–	++	–	–	–	++	∓
27..	–	–	++	+	+	+	–	–	–	+	±	–	–	+++	+	–	+	–	–	–	+	±
29..	–	–	+++	+	+	+	–	–	–	+	±	–	–	+++	++	–	++	+	–	–	+	±
30..	–	–	++	+	–	++	–	–	–	+	±	–	–	++	++	–	++	+	–	–	+	±
VIII— 4..	–	–	++	+	–	++	–	–	–	+	±	–	–	+++	++	–	++	++	–	–	+	±
6..	–	–	++	+	–	+	–	–	–	+	±	–	–	+++	++	–	++	++	–	–	+	±
9..	–	–	++	+	–	+	–	–	–	+	±	–	–	+++	++	–	++	++	–	–	+	±
12..	–	–	++	+	–	+	–	+	–	+	±	–	–	+++	++	–	++	++	–	–	–	±
13..	–	–	++	+	–	++	–	–	–	–	±	–	–	++	++	–	++	++	+	–	–	±
14..	–	+	++	+	–	++	–	–	–	–	±	–	–	++	++	+	++	–	–	–	+	±
16..	–	+	++	+	–	++	+	+	–	++	±	–	–	++	++	–	++	–	+	–	++	±
18..	–	+	++	+	–	++	–	–	–	+	±	–	++	+	+	–	+	–	–	–	++	±
20..	–	++	–	–	–	–	–	–	–	++	+	–	++	–	–	–	–	–	+	–	++	+
23..	–	++	–	–	–	?	–	–	–	+	+	++	++	+	–	–	?	–	–	–	++	+
24—	–	++	+	–	–	+	–	–	–	+	+	–	++	+	–	–	–	–	–	–	++	+
27..	–	+	+	–	–	–	–	–	–	+	+	–	++	+	–	–	–	–	–	–	+	+
30..	–	+	++	+	–	–	–	–	–	+	+	–	++	+	–	–	+	–	–	–	++	+

TABLE II—Continued.

Fermentation tubes.				Urine.			Milk.			Diet.	Remarks.
Dextrose.	Lactose.	Saccharose.	Type of growth.	Indican.	Indolacetic acid.	Millon's	Coagulated.	Gas.	Peptonization.		
40	85	30	±	+	–	+++				Meat	Feces dark brown tarry
95	80	95	++	++	–	++	++	80	+	"	"
80	60	70	+	∣	–	+++				"	"
100	100	75	++	–	–	+++				"	"
70	70	80	++	++	–	+++				"	"
75	70	85	++							"	"
70	80	80	++	+++	–	+++	++	100	+	"	"
60	85	65	++							"	"
90	60	20	++	++		+++	++	100	+	"	"
55	60	85	++							"	"
55	80	90	++								"
80	50	40	++	++	–	+++	++	90	+	Milk	Feces light brown, some mucus—soft
20	25	25	++	∣	–	+++					Feces light brown—watery
25	25	30	++	∣	–	+++				"	Feces light brown—soft
20	30	30	++							"	"
25	10	10	+	–	–	–	+	60	+	"	"
20	10	10	+	–	–	–	–			"	"
3	20	15	+							"	*Feces gray brown-soft*
10	10	10	+	∣	–	–	+	5		"	"
10	15	15	+							"	"
10	10	2	+	–	–	–	+	–	+	"	"

TABLE III.

Kitten C.

DATE.	FECES. GRAM STAIN. Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.
VII—17.......	—	∣	∣	∣	—	+	+	—	—	∣	±
VII—18.......	—	∣	∣	∣	—	+	+	—	—	∣	±
VII—20.......	—	?	+	+	—	+	+	+	—	∣	±
VII—23.......	—	—	++	+	+	+	—	—	—	+	±
VII—25.......	—	—	++	+	∣	+	+	—	—	—	±
VII—26.......	—	—	++	+	+	+	±	—	—	+	±
VII—27.......	—	—	++	+	∣	+	—	—	∣	+	±
VII—30.......	—	—	++	—	—	+	+	—	∣	+	±
VII—31.......	—	—	++	+	+	++	+	—	+	+	±
VIII— 1.......	—	—	++	+	—	++	+	—	—	+	±
VIII— 2.......	—	—	++	+	—	++	+	—	—	+	±
VIII— 4.......	—	—	++	+	—	+	∣	—	—	+	±
VIII— 6.......	—	—	++	+	∣	++	∣	—	—	+	±
VIII— 9.......	—	—	++	+	+	++	∣	—	—	+	±
VIII—11.......	—	—	++	+	∣	++	∣	—	—	+	±
VIII—14.......	—	—	++	+	+	++	+	—	—	+	±
VIII—18.......	∣	—	++	+	—	++	∣	—	—	∣	±
VIII—20.......	?	++	∣	∣	—	∣	—	—	—	++	+
VIII—23.......	—	++	∣	∣	—	∣	—	—	—	++	+
VIII—24.......	—	+	∣	∣	—	∣	—	—	—	+	+
VIII—26.......	—	++	—	—	—	—	—	—	—	+	+
VIII—28.......	—	++	—	—	—	—	—	—	—	+	+
VIII—30.......	—	++	—	—	—	—	—	—	—	+	+

DATE.	FERMENTATION TUBE SEDIMENTS. GRAM STAIN. Bifidus.	Acidiphilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.
VII—17.......	—	?	+	+	—	+	∣	—	—	∣	±
VII—18.......	—	?	+	+	—	+	+	—	—	∣	±
VII—20.......	—	—	++	+	—	++	∣	+	+	+	±
VII—23.......	—	—	++	+	—	++	∣	+	+	+	±
VII—25.......	—	—	+++	+	—	++	∣	+	∣	+	±
VII—26.......	—	—	+++	+	—	++	+	—	∣	+	±
VII—27.......	—	—	+++	+	—	++	+	—	∣	+	±
VII—30.......	—	—	+++	+	—	++	+	—	∣	+	±
VII—31.......	—	—	+++	+	—	++	++	—	∣	+	±
VIII— 1.......	—	—	+++	+	—	++	+	—	∣	+	±
VIII— 2.......	—	—	+++	+	—	++	+	—	∣	+	±
VIII— 4.......	—	—	+++	+	—	++	+	—	∣	∣	±
VIII— 6.......	—	—	+++	++	—	++	+	—	∣	∣	±
VIII— 9.......	—	—	+++	++	+	++	++	—	∣	+	±
VIII—11.......	—	—	+++	++	∣	++	++	—	—	+	±
VIII—14.......	—	—	+++	++	—	++	+	—	—	+	±
VIII—18.......	±	+	++	∣	—	+	+	—	—	—	±
VIII—20.......	—	+	++	∣	+	+	—	—	—	+	±
VIII—23.......	∣	+	+	∣	—	+	—	—	—	+	±
VIII—24.......	—	+	+	∣	—	∣	—	—	—	+	±
VIII—26.......	+	+	∣	—	—	∣	—	—	—	++	+
VIII—28.......	+	+	—	—	—	—	—	—	—	+	+
VIII—30.......											

TABLE III—*Continued*

FERMENTATION TUBES.				URINE.			MILK.			ACIDOPHILES, ACETIC ACID BROTH.			DIET.	REMARKS.
Dextrose.	Lactose.	Saccharose.	Type of growth.	Indican.	Indolacetic Acid.	Millon's.	Coagulated.	Gas.	Reaction.	5 per cent.	10 per cent	20 per cent.		
60	30	90	++	++	–	+++	+	*p. c.*		++	++	+	Meat	
70	35	65	++				+++	95	+				"	
80	85	95	++	+	–	+++				++	+	–	"	
95	65	40	++	+	–	+++							"	
90	70	60	++	+	–	+++							"	
35	80	70	++	+	–	+++	++	85	++	++	+	–	"	
55	70	80	++	+++	–	+++	++	100	++				"	
60	65	45	++	++	–	++	++	100	++	+	–	–	"	
80	40	65	++	+	–	++	++	100	++				"	
80	65	45	++	+	–	++							"	
100	95	90	++	+	–	++							"	
50	70	100	++	+	–	+							"	
80	90	85	++	+	–	+++							"	
50	55	65	++	++	–	+++							"	
80	90	80	++										"	
90	60	85	++	++	–	+++							Milk and dextrose	
20	15	20	++	–	–	–	++	20	+					
25	35	15	++	–	–	–							"	
30	20	10	++	–	–	–							"	
20	15	30	++	–	–	–	+	5	++				"	
0	3	2	+	–	–	–							"	
0	2	1	+	–	–	–	+	–	++				"	
0	0	0	+										"	

TABLE IV.

Monkey 1 (Control Monkey)

DATE.	FECES. GRAM STAIN.											FERMENTATION TUBES SEDIMENTS. GRAM STAIN.										
	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.
VI— 8	−	−	+	+	+++	+	+	−	−	−	±	−	−	++	+	−	+	+	−	+	−	±
9	−	−	+	+	++	+	+	−	−	±	+	−	−	−	−	−	+	+	−	−	−	±
10	−	−	+	+	++	+	+	−	−	±	±	−	−	−	−	−	+	+	+	+	++	±
11	−	−	+	+	+	+	+	−	−	±	±	−	−	++	++	−	−	+	+	+	+	±
12	−	−	+	+	−	+	+	−	−	−	±	−	−	++	+	+	+	+	−	−	±	±
13	−	−	+	+	−	+	++	−	−	+	±	−	−	++	+	−	+	+	−	−	+	±
14	−	−	+	+	+	+	++	−	−	+	±	−	−	++	+	−	−	+	−	−	+	+
15	−	−	+	+	+	+	+	−	−	+	±	−	−	++	+	−	+	+	−	−	+	±
16	−	−	+	+	+	+	−	−	−	+	±	−	−	++	−	−	+	−	+	−	++	±
17	−	−	+	+	−	+	−	−	−	+	+	−	−	++	−	−	+	−	+	+	+	±
18	−	−	−	+	+	−	+	−	−	+	±	−	?	+	+	−	+	+	−	+	++	±
19	−	−	+	+	−	+	−	−	−	+	±	−	−	+	+	−	+	−	−	−	++	±
20	−	−	+	+	−	+	−	−	−	+	±	−	−	+	+	−	+	−	−	−	++	+
21	−	−	+	+	−	+	+	−	−	−	±	−	−	+	+	−	+	−	+	−	+	±
23	−	−	−	+	−	+	+	−	−	−	±	−	−	+	+	−	+	+	+	+	+	±
24	−	−	+	+	−	+	+	−	−	−	±	−	−	+	+	−	++	+	+	−	±	±
25	−	−	+	+	−	+	+	−	−	−	±	−	−	+	+	−	++	+	+	−	±	+
26	−	−	++	+	−	++	+	−	−	−	±	−	−	+	−	−	+	++	+	+	±	
27	−	−	+	+	+	+	−	−	−	−	±	−	−	+	+	−	−	+	+	−	+	±
28	−	−	++	+	+	+	−	−	−	+	±	−	−	+	−	−	+	++	+	−	−	±
29	−	−	++	+	+	+	−	−	−	−	±	−	−	+	−	−	+	++	+	+	−	±
30	−	−	++	+	−	+	−	−	−	−	±	−	−	+	−	−	+	++	−	+	+	±
VII— 1	−	−	++	+	−	+	++	−	−	+	±	−	−	++	−	−	+	+	+	−	−	±
2	−	−	++	+	−	+	++	−	−	−	±	−	−	++	−	−	+	+	++	−	+	±
3	−	−	++	+	−	+	+	+	−	++	±	−	−	+	−	−	+	++	+	−	+	±
4	−	−	++	+	−	+	−	−	−	+	±	−	−	+	+	−	+	+	+	−	+	±
5	−	−	++	+	−	+	+	−	−	+	±	−	−	++	+	−	+	++	+	−	+	±
VII— 6	−	?	++	+	−	+	+	−	−	−	±	−	+	+	−	−	+	+	−	+	+	±
7	−	?	+	−	−	+	−	−	−	−	±	+	+	+	−	−	++	+	+	+	++	±
8	−	−	+	−	−	+	−	−	−	+	±	−	+	+	−	−	++	+	+	+	++	±
9	−	+	+	+	−	+	+	−	−	+	±	−	+	+	−	−	++	+	+	+	++	±
10	−	−	++	+	−	+	−	−	+	+	±	−	−	++	+	−	+	−	−	+	+	±

TABLE IV.—Continued.

Fermentation tubes.				Urine.			Milk.			Acidophiles; acetic acid broth.			Diet.	Remarks.
Dextrose.	Lactose.	Saccharose.	Type of growth.	Indican.	Indolacetic acid.	Millon's.	Coagulated.	Gas.	Peptonized.	5 per cent.	10 per cent.	20 per cent.		
60	70	40	++										Mixed diet; bread & bananas.	
80	100	100	++										" "	
90	85	80	++				+	80	+	+	+	—	" "	
75	30	50	++				+	90	+				" "	
85	55	90	++				+	60	+				" "	
90	80	90	++				+	5	—				" "	
80	70	90	++				+	10	—				" "	
30	20	30	++				+	5	—				" "	
80	95	70	++				+	5	—				" "	
25	30	80	++				+			++	—	—	" "	
25	30	20	++							+	—	—	" "	
20	30	10	++				+	5	—				" "	
25	30	20	++				+	5	—				" "	
30	10	20	++							+	—	—	" "	
30	20	20	++							+	—	—	" "	
40	35	20	+++				+	20	—	—	—	—	" "	
65	50	65	++				—	—	—	—	—	—	" "	
40	50	65	+				+	10	—	—	—	—	" "	
30	95	90	++				+	15	—	—	—	—	" "	
85	80	95	++							—	—	—	" "	
85	80	90	++							—	+	+	" "	
60	85	95	++										" "	
10	5	15	+										" "	
40	40	50	+										" "	
10	10	12	++							+	+	+	" "	
40	35	30	++										" "	
10	15	10	++										" "	
1	3	2	++	—	?	?	+	20	+	+	+	+	" "	
10	20	2	++	—	—	—							" "	
2	3	1	++	—	—	—							" "	
2	1	3	++										" "	
40	45	40	++										" "	

TABLE V.

Monkey II.

Date.	Feces. Gram stain.											Fermentation tube sediments. Gram stain.										
	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.
VI—7.....	—	+	—	+	—	—	+	+	—	—	±	—	—	—	—	—	—	+	—	—	—	+
8.....	—	+	—	+	+	—	+	+	—	—	±	—	+	—	—	—	—	+	+	+	+	+
9.....	—	+	+	+	—	+	+	—	—	+	±	—	+	+	—	—	—	—	—	—	+	+
10.....	—	—	+	+	+	+	+	—	—	+	±	—	—	+	—	—	+	—	+	+	+	+
11.....	—	—	+	+	—	+	+	—	+	+	±	—	—	+	—	—	+	+	+	+	++	+
12.....	—	—	+	+	—	+	+	—	—	—	±	—	—	+	—	—	+	+	—	+	+	+
13.....	—	—	+	+	—	+	+	—	—	—	±	—	—	+	—	—	—	++	—	—	+	+
14.....	—	—	++	+	—	+	++	—	—	—	±	—	—	+	+	—	+	++	—	+	+	+
15.....	—	—	++	—	—	+	++	—	—	—	+	—	—	+	+	—	+	++	—	—	+	+
16.....	—	+	++	+	—	+	+	—	—	+	±	—	—	+	+	—	+	—	—	—	+	+
17.....	—	+	++	+	+	+	+	—	—	+	±	—	—	+	+	—	+	+	+	+	+	±
18.....	—	—	+	+	—	+	+	—	+	+	±	—	+	—	+	—	—	+	—	+	+	±
19.....	—	—	+	+	—	—	+	—	+	+	±	—	+	+	+	—	—	+	—	+	+	+
20.....	—	—	+	+	—	+	+	—	+	+	±	—	—	+	+	—	+	+	—	+	+	+
22.....	—	—	?	+	—	—	—	—	—	++	+	++	—	+	—	+	—	—	+	—	+	+
23.....	—	—	?	+	—	—	—	—	—	++	+	—	+	?	+	—	—	+	—	—	+	+
24.....	—	—	?	+	—	—	—	—	—	++	+	—	+	—	+	—	+	+	—	—	+	+
25.....	—	—	—	+	—	—	—	—	—	++	+	—	+	—	—	—	—	++	—	—	+	+
26.....	—	+	—	+	—	—	—	—	—	++	+	—	+	—	—	—	—	—	—	—	++	+
27.....	—	+	—	—	—	?	—	—	—	++	+	—	+	—	—	—	—	+	—	—	—	+
28.....	—	+	—	—	—	—	—	—	—	+	+	+	+	—	—	—	—	—	—	—	++	+
29.....	—	+	—	—	—	—	—	—	—	+	+	++	+	—	—	—	—	—	+	—	++	+
30.....	—	+	—	+	—	—	—	—	—	+	+	—	+	—	—	—	—	—	—	—	++	+
VII—1.....	—	+	—	+	—	—	+	—	—	+	+	—	+	—	—	—	—	—	—	—	++	+
2.....	—	+	—	+	—	+	—	—	+	+	+	++	++	—	—	—	—	—	—	—	+	+
3.....	—	+	+	—	—	—	—	—	+	+	±	++	++	—	—	—	—	?	—	—	—	+
4.....	—	+	—	—	—	—	—	—	—	+	+	++	+	—	—	—	—	—	—	—	+	+
5.....	—	+	—	—	—	—	—	—	—	+	+	++	+	—	—	—	—	—	—	—	+	+
VII—6.....	—	+	—	—	—	+	+	—	—	—	+	++	+	—	—	—	+	—	—	—	+	+
7.....	—	+	—	—	—	—	—	—	—	+	+	++	+	—	—	—	—	—	—	—	+	+
8.....	—	+	—	—	—	—	—	—	—	+	+	—	+	—	—	—	+	—	—	—	++	+
9.....	—	+	+	—	—	+	—	—	—	+	+	+	+	—	—	—	+	—	—	—	++	+
10.....	—	—	+	—	—	+	—	—	—	+	+	—	—	+	+	—	+	—	—	—	+	+
11.....	—	—	+	—	—	+	+	—	—	—	±	—	—	++	+	—	++	—	—	—	+	±
12.....	—	+	+	—	—	+	+	—	—	+	±	—	—	++	+	—	+	—	—	—	—	+
13.....	—	—	+	+	—	++	+	—	—	—	±	—	—	++	+	—	+	—	—	—	—	±
14.....	—	—	+	—	—	+	—	—	—	—	±	—	—	++	+	—	+	—	—	—	—	±
15.....	—	—	+	—	—	++	—	—	—	—	±	—	—	++	+	—	+	—	—	—	—	±
16.....	—	—	+	+	—	±	—	—	—	—	±	—	—	++	+	—	+	—	—	—	+	±
17.....	—	—	—	—	—	—	—	—	—	—	±	+	—	++	+	—	+	—	—	—	—	±
18.....	—	?	+	—	—	—	—	—	—	—	±	—	—	++	++	—	+	—	—	—	+	±
19.....	—	—	+	—	—	—	—	—	—	—	±	—	?	++	++	—	+	—	—	—	—	±

TABLE V.—Continued.

FERMENTATION TUBES.				URINE.			MILK.			ACIDOPHILES ACETIC ACID BROTH.			DIET.	REMARKS.
Dextrose.	Lactose.	Saccharose.	Type of growth.	Indican.	Indolacetic Acid.	Millon's.	Coagulated.	Gas.	Peptonized.	5 per cent.	10 per cent.	20 per cent.		
2	0	0	+										Bread and bananas.	
0	1	2	+										"	
10	9	5	+										"	
12	5	6	+				+	20	+				"	
0	7	7	+				+	—	+				"	
0	2	2	+				+	—	+				"	
3	9	9	+				+	—	+				"	
0	4	5	+				+	5	+				"	
0	4	5	+				+	5	+				"	
0	1	4	+				+	10	+				"	
45	60	60	++				+	30	+				"	
3	0	2	+				+	—	+				"	
2	0	0	+				+	—	+				Milk and dextrose	
2	1	5	+	—			+	—	+				"	
0	0	0	+	—			+	|	+				"	
0	7	1	+	—			+	—	+				"	
2	1	4	+	—			+	—	+	++	++	+	"	
0	1	0	+	—			+	—	+	++	++	+	"	
2	0	0	+	—			+	—	+	++	++	+	"	
4	3	3	+	—			+	—	+	++	+	+	"	
8	2	3	+	—			+	—	+	++	|	|	"	
8	1	1	+	—			+	—	+	++	++	++	"	
1	2	1	+				+	—	+				"	
6	2	3	+										"	
0	1	1	+										"	
0	0	0	+	—			+	—	+				"	
0	0	1	+										"	
3	1	1	+										"	
0	0	0	+	|	—	—	+	10	+	+	+	+	"	
0	1	3	+	+	—	+							"	
1	3	3	+	—	—	|							"	
10	10	5	+							++	++	+	"	
15	15	10	+										"	
25	15	10	++										"	
20	20	20	++							++	|	—	Eggs.	
35	50	50	++										"	
50	60	55	++										"	
65	50	50	++							+	—	—	"	
20	46	48	++										"	
5	10	12	++	+++	—	+++				+	—	—	"	
45	50	35	++	+	—	++				+	—	—		
40	55	50	++							|	—	—		

TABLE V.—*Continued.*

DATE.	FECES GRAM STAIN.											FERMENTATION TUBE SEDIMENTS. GRAM STAIN.										
	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms	Type.	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.
VII—20.....	—	?	+	+	—	+	+	—	—	|	±	—	+	++	++	—	++	+	—	—	+	±
21.....	—	—	+	+	—	+	—	—	—	+	±	—	+	|	|	—	+	—	—	—	++	±
22.....	—	+	|	+	—	|	—	—	—	++	±	++	+	|	|	—	|	—	—	—	++	+
23.....	—	+	|	|	—	—	|	—	—	++	+	++	++	—	—	—	?	—	—	—	++	+
24.....	—	+	|	+	—	+	|	—	—	++	+	—	+	|	+	—	+	—	—	—	+	±
25.....	—	|	+	+	—	+	—	—	—	+	±	—	+	+	+	—	+	—	—	—	+	±
26.....	—	+	+	+	—	+	—	—	—	++	+	+	+	|	—	|	—	—	—	—	+	±
27.....	—	+	+	+	—	+	+	—	—	++	+	—	++	+	|	—	—	—	—	—	+	+
28.....	—	+	—	—	—	|	—	—	—	++	+	+	++	|	|	—	—	—	—	—	++	+
29.....	—	+	—	—	—	|	—	—	—	++	+	+	++	—	—	—	—	—	—	—	++	+
30.....	—	+	—	—	—	—	—	—	—	++	+	+	+	—	—	—	—	—	—	—	++	+
31.....	—	+	—	—	—	|	—	—	—	+	+	—	+	—	—	—	—	—	—	—	++	+
VIII-1.....	—	+	—	—	—	|	—	—	—	+	+	—	+	—	—	—	—	—	—	—	+	+
2.....	—	+	—	—	—	—	—	—	—	+	+	—	+	|	|	—	|	—	—	—	++	+
3.....	—	+	?	|	—	|	—	—	—	|	+	—	+	?	|	—	|	|	—	|	+++	+
4.....	—	+	?	|	—	|	+	—	—	|	+	+	+	|	|	—	|	|	—	|	+++	+
5.....	—	+	+	+	—	+	|	—	—	|	+	++	+	|	|	—	|	—	—	—	++	+
6.....	—	+	|	|	—	|	|	—	—	|	+	+	+	|	|	—	|	—	—	—	++	+
7.....	—	+	|	|	—	?	—	—	—	|	+	+	+	?	|	—	|	—	—	|	++	+
8.....	—	+	|	|	—	?	—	—	—	|	+	—	+	—	|	—	|	—	—	—	+++	+
9.....	—	+	—	|	—	—	—	—	—	|	+	+	++	—	|	—	|	—	—	—	++	+
10.....	—	+	—	|	—	—	—	—	—	++	+	+	++	—	|	—	|	|	—	—	++	+
11.....	—	+	+	+	—	+	|	—	—	|	±	++	+	++	+	—	+	|	—	—	+	±
12.....	—	+	+	+	+	+	|	—	—	|	±	+	|	++	+	+	+	|	—	—	++	+
13.....	—	+	+	+	|	+	+	—	—	+	±	—	|	++	++	|	+	+	—	—	+	±
14.....	—	|	++	+	—	+	+	—	—	|	±	—	+	++	+	—	++	|	—	—	+	±
15.....	—	|	++	+	—	+	|	—	—	+	±	—	|	++	+	—	++	|	—	—	+	±
16.....	—	|	++	+	—	+	|	—	—	+	±	—	—	++	+	—	++	+	—	—	|	±
17.....	—	+	++	+	—	+	|	—	—	++	±	—	—	++	+	+	—	+	—	+	|	±
18.....	—	|	++	+	—	+	|	—	+	+	±	—	—	++	++	—	+	|	—	—	|	±
19.....	—	|	+	+	—	+	+	—	—	+	±	—	+	+	+	—	+	|	—	—	+	±
20.....	—	+	+	+	—	+	+	—	—	+	±	—	+	++	+	—	+	|	—	—	+	±
21.....	—	+	+	+	—	+	|	—	—	|	+	—	+	+	|	—	|	|	—	—	++	+
22.....	—	+	|	|	—	+	—	—	—	+	+	—	+	+	?	—	|	—	+	—	++	+
23.....	—	+	|	?	—	—	—	—	+	+	+	—	+	+	—	—	—	—	—	—	++	+
24.....	—	+	|	?	—	—	—	—	—	++	+	—	+	+	—	—	—	—	—	—	++	+
25.....	—	+	+	|	—	+	—	—	—	++	+	—	++	+	—	—	—	—	—	—	++	+
26.....	—	+	+	+	—	—	|	—	—	++	+	—	++	+	—	—	—	—	—	—	+	+
27.....	—	+	+	+	—	—	+	—	—	+	+	—	++	+	—	—	—	—	—	—	++	+
28.....	—	+	+	|	—	—	+	—	—	+	+	—	++	|	—	—	—	—	—	—	++	+
29.....	—	+	|	—	—	—	|	—	—	+	+	—	++	+	—	—	—	—	—	—	++	+
30.....	—	+	|	—	—	—	|	—	—	+	+	—	++	|	—	—	—	—	—	—	++	+

TABLE V.—Continued.

Fermentation tubes.				Urine.			Milk.			Acidophiles acetic acid broth.			Diet.	Remarks.
Dextrose.	Lactose.	Saccharose.	Type of growth.	Indican.	Indolacetic acid.	Millon's.	Coagulated.	Gas.	Peptonized.	5 per cent.	10 per cent.	20 per cent.		
80	70	70	++	∣	–	++	+	35	+	∣	–	–	Milk and dextrose	
0	1	2	++				++	15	+	++	++	+	“	
2	1	2	+	–	∣	–				++	++	+	“	
0	0	2	+							++	++	+	“	
2	8	4	++	–	–	–							“	
0	30	0	+	–	–	–							“	
4	2	1	+				++	?gas	+				“	
10	0	3	+										“	
0	3	0	+	–	–	–							“	
2	3	1	+				++	–	+				“	
2	9	3	+	∣	–	–	++	–	+				“	
1	3	1	+				++	–	+				“	
4	2	1	+										“	
2	3	0	+										“	
3	5	0	+	–	∣	–							“	
5	0	2	+	–	–	–							“	
25	15	20	++	–	–	–							“	
0	2	0	+	+									“	
0	0	0	∣	∣	–	–							“	
2	5	2	++	–	∣	–							“	
1	2	0	++										“	
2	5	2	+	++	–	∣							Eggs.	
20	20	20	++										“	
55	60	50	++	++	–	+							“	
50	40	35	++										“	
45	45	40	++	++	–	++							“	
50	70	75	++	++	–	++							“	
50	60	50	++										“	
60	50	50	++	++	–	++							“	
30	55	30	++	+	–	+							“	
20	15	7	++										“	
25	20	20	++	+	–	+							Milk and dextrose	
10	7	4	++										“	
1	3	2	+	∣	–	+							“	
2	1	5	+	–	–	+							“	
3	4	2	+	–	–	–							“	
25	20	10	++										“	
0	2	1	+										“	
0	3	2	+										“	
0	2	0	+										“	
3	4	2	+										“	
0	1	0	+										“	

TABLE VI.

Monkey III.

DATE.	FECES. GRAM. STAIN.											FERMENTATION TUBE SEDIMENTS. GRAM STAIN.										
	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.	Bifidus.	Acidophilus	Large subtiloid	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.
VI- 7	—	+	—	+	—	—	+	—	—	+	±	—	+	—	—	—	—	+	—	—	—	+
8	—	+	—	+	—	+	—	—	—	+	±	—	+	+	—	—	—	++	—	—	+	+
9	—	+	+	—	—	+	+	—	—	+	±	—	+	+	+	—	+	+	—	—	+	+
10	—	+	—	—	—	—	+	—	—	+	+	—	+	+	+	—	+	+	—	—	+	+
11	—	+	—	—	—	—	+	—	—	+	+	—	+	+	—	—	—	+	—	—	+	+
12	—	+	—	—	—	—	+	—	—	+	+	—	+	—	—	—	—	+	—	—	+	+
13	—	+	+	—	—	—	+	—	—	+	+	—	+	—	—	—	+	+	—	—	+	+
14	—	+	—	—	—	—	+	—	—	++	+	—	+	—	—	—	+	+	—	—	+	+
15	—	+	+	—	—	—	+	—	—	+	+	—	+	—	—	—	—	+	—	—	++	+
16	—	+	—	—	—	—	+	—	—	+	+	—	+	—	—	—	—	+	—	—	+	+
17	—	+	—	—	—	—	+	—	—	++	+	—	+	—	+	—	+	—	—	—	+	+
18	—	+	—	—	—	—	+	—	—	+	+	—	+	—	—	—	—	+	—	—	+	+
19	—	+	—	—	—	—	+	—	—	+	+	—	+	—	—	—	—	+	—	—	+	+
20	—	+	—	—	—	+	+	—	—	+	+	—	+	—	—	—	—	—	—	—	+	+
22	—	+	—	—	—	—	—	—	—	+	+	—	+	++	+	—	+	+	—	—	+	±
23	—	+	+	+	—	+	+	—	—	+	±	—	—	++	+	—	+	—	—	—	+	±
24	—	—	+	+	—	+	+	—	+	—	±	—	—	++	+	—	++	+	+	+	+	±
26	—	—	+	+	—	+	—	—	—	—	±	—	—	++	++	—	+	—	—	—	—	±
27	—	—	+	—	—	++	—	—	—	—	±	—	—	++	++	+	+	?	—	—	—	±
28	—	—	+	—	+	+	—	—	—	+	±	—	—	++	+	—	+	—	—	—	—	±
29	—	—	+	—	—	+	—	—	—	—	±	—	—	++	+	—	+	—	—	—	+	±
30	—	—	+	+	—	+	—	—	—	+	±	—	—	++	++	+	—	—	—	—	—	±
VII- 1	—	—	+	+	—	+	—	—	—	—	±	—	—	++	+	—	+	—	—	—	+	±
2	—	—	+	+	—	+	—	—	—	+	±	—	—	++	+	—	+	—	—	—	+	±
3	—	—	+	+	—	+	—	—	—	—	±	—	—	++	+	—	+	—	—	—	+	±
4	—	—	+	+	—	+	—	—	—	—	±	—	—	++	+	—	+	—	—	—	+	±
5	—	—	+	+	—	+	+	—	—	+	±	—	—	+	+	—	—	—	—	—	+	±

TABLE VI.—Continued.

FERMENTATION TUBES.				URINE.			MILK.			ACIDOPHILES: ACETIC ACID BROTH.		DIET.	REMARKS.
Dextrose.	Lactose.	Saccharose.	Type of growth.	Indican.	Indolacetic acid.	Millon's.	Coagulated.	Gas.	Peptonized.				
0	2	1	+									Milk and Dextrose.	
1	2	0	+									"	
1	5	5	+									"	
8	5	8	+	—			+	20				"	
3	5	2	+									"	
3	5	3	+									"	
1	3	2	+									"	
0	7	1	+	—			+	10	+			"	
7	7	9	+	—			+	5	+			"	
2	2	3	+	—			+	20	+			"	
5	5	2	+	—			+	5	+			"	
1	5	1	+	—			+	10	+			"	
3	2	3	+	—			+	5	+			"	
2	3	3	+	—			+	5	+			Eggs.	
55	40	30	++	—			+	20	+			"	
60	50	40	++	—			+	20	+			"	
50	50	60	++	—			+	40	+			"	
60	70	55	++				+	95	+			"	
50	60	60	++	+		+	+	80	+			"	
80	70	75	++	+		+	+	90	+			"	
70	70	80	++	+	—	++						"	
65	80	70	++	++	—	++						"	
65	65	70	++	+	—	++						"	
50	70	70	++	+	—	+						"	
80	60	70	++	+	—	+						"	
60	75	70	++	+	—	+						"	
85	75	80	++									"	

TABLE VII.

Monkey IV.

Date.	Feces. Gram stain.											Fermentation tube sediments. Gram stain.										
	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms	Type.	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.
VI— 7...	−	−	+	+	−	+	+	−	−	−	±	−	−	+	+	−	+	+	−	−	+	+
8...	−	−	+	+	−	+	+	−	−	−	±	−	−	+	+	+	+	+	−	−	−	±
9...	−	−	+	+	−	+	−	−	−	−	±	−	−	−	+	+	−	+	+	−	−	±
10...	−	−	+	+	−	+	−	−	−	−	±	−	−	+	−	−	−	+	−	−	−	±
11...	−	−	+	+	−	+	−	−	−	−	±	−	−	+	−	−	+	+	−	−	+	±
12...	−	−	+	−	−	+	−	−	−	+	±	−	−	+	−	−	+	++	−	−	+	+
13...	−	−	+	−	−	+	−	−	−	+	±	−	−	++	−	−	+	++	−	−	+	±
14...	−	−	+	−	−	−	−	−	−	+	±	−	−	++	+	−	−	+	−	−	−	±
15...	−	−	+	+	−	+	−	−	−	−	±	−	−	+	+	−	−	++	+	−	−	±
16...	−	−	+	+	−	+	−	−	−	+	±	−	−	+	−	−	+	+	−	−	+	±
17...	−	−	+	+	−	+	−	−	−	+	±	−	−	+	−	−	+	+	−	−	−	±
18...	−	−	+	−	−	+	−	−	−	+	±	−	+	+	−	+	−	−	−	−	+	±
19...	−	−	+	−	−	+	+	−	−	+	±	−	−	+	+	−	+	−	−	−	+	±
20...	−	−	+	−	−	+	−	−	−	+	±	−	−	+	+	−	+	+	−	−	+	±
23...	−	−	+	−	−	+	+	−	−	−	±	−	−	++	+	−	+	++	−	+	+	±
24...	−	−	+	−	+	++	+	−	−	−	±	−	−	+		−	−	++	+	+	+	±
25...	−	−	+	+	−	++	+	−	−	−	±	−	−	+	+	−	+	++	−	−	+	±
26...	−	−	+	++	−	++	−	−	−	+	±	−	−	++	−	−	−	−	−	−	+	±
27...	−	−	++	−	−	+	+	−	−	−	±	−	−	++	−	−	−	−	−	−	−	±
28...	−	−	++	−	−	+	−	−	−	−	±	−	−	++	−	−	−	++	−	−	−	±
29...	−	−	+	−	−	+	−	−	−	−	±	−	−	++	−	−	−	++	−	−	+	±
30...	−	−	+	−	−	+	−	−	−	−	±	−	−	++	−	−	++	−	−	−	++	±
VII—1...	−	−	−	−	−	+	−	−	−	−	±	−	−	++	−	−	++	−	−	−	++	±
2...	−	−	+	−	−	+	−	−	−	−	±	−	+	++	−	−	++	−	−	−	+	±
3...	−	−	+	+	−	+	+	−	−	+	±	−	−	++	−	−	++	−	−	−	+	±
4...	−	−	−	−	−	+	−	−	−	+	±	−	−	++	−	−	++	−	−	−	+	±
5...	−	−	−	+	−	+	+	−	−	−	±	−	−	++	−	+	−	−	−	−	+	±
VII- 6...	−	−	+	+	+	−	+	−	−	−	±	−	−	++	+	−	+	−	−	−	+	±
7...	−	−	+	+	−	+	−	−	−	+	±	−	−	++	+	−	+	−	−	+	+	±
8...	−	−	+	+	−	+	−	−	−	−	±	−	+	++	+	−	+	−	−	+	+	±
9...	−	−	+	+	−	+	−	−	−	+	±	++	++	+	+	−	−	+	−	−	+	±
10...	−	+	+	+	−	+	−	−	−	+	±	++	+	+	−	−	+	−	−	−	−	±
11...	−	−	+	+	−	+	−	−	−	−	±	+	+	+++	+	−	+	−	−	−	−	±
12...	−	−	+	+	−	+	−	−	−	−	±	+	+	++	−	−	−	−	−	−	+	±
13...	−	−	+	−	−	+	+	−	−	−	±	+	+	+	−	−	−	−	−	−	+	±
14...	−	+	−	−	−	−	+	−	−	+	±	+	+	−	−	−	−	−	−	−	+	+
15...	−	+	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	+	+
16...	−	+	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	+	+
17...	−	+	−	−	−	+	−	−	−	+	+	+	+	−	−	−	−	−	−	−	+	+
18...	−	+	−	−	+	−	−	−	−	+	+	−	+	+	+	−	+	−	−	−	+	+

TABLE VII.—Continued.

Fermentation tubes.				Urine.			Milk.			Acidophiles, acetic acid broth.			Diet.	Remarks.
Dextrose.	Lactose.	Saccharose.	Type of growth.	Indican.	Indolacetic acid.	Millon's.	Coagulated.	Gas.	Peptonized.	5 Per cent.	10 Per cent.	20 Per cent.		
2	4	3	++										Milk and dextrose.	
1	3	2	++										"	
3	5	8	++										"	
2	5	3	+	−			+	5	+				"	
4	5	3	+	−			+	5	+				"	
2	5	2	+	−			+	20	+				"	
7	5	6	+	−			+	10	+				"	
5	6	5	+	−			+	40	+				"	
5	3	9	+	−			+	20	+				"	
2	9	8	+	−			+	10	+				"	
4	2	4	+	−			+	3	+				"	
6	2	1	+	−			+	5	+				"	
3	4	2	+	−			+	5	+				"	
3	3	2	+	−			+	5	+				Eggs.	
80	75	95	++	−			+	5	+				"	
70	70	85	++	−			+	5	+	+	+	−	"	
25	60	65	++	−			+	80	+	+	+	−	"	
60	55	55	++	−			+	70	+	+	+	−	"	
70	70	60	++	−			+			+	−	−	"	
40	50	40	++	−						+	−	−	"	
60	60	50	++	−						+	−	−	"	
60	70	60	++	+									"	
60	80	60	++	+									"	
85	85	75	++	+									"	
80	85	80	++	+									"	
80	85	70	++	+									"	
90	90	80	++										"	
80	80	70	++	+	−	+	+	60	+	+	−	−	"	
85	70	80	++										"	
70	80	70	++	++	−	++							"	
75	80	70	++	+						++	+	+	"	
40	50	40	++										"	
35	40	40	++	+	−	++							"	
30	25	30	++							++	+	+	"	
25	20	35	++										Milk and dextrose.	
0	0	4	+										"	
0	0	1	+							++	++	+	"	
0	2	2	+							++	++	+	"	
3	2	2	+										"	
3	4	3	+	−	−	−				++	++	++	"	

TABLE VII.—Continued.

DATE.	FECES. GRAM STAIN.											FERMENTATION TUBE SEDIMENTS. GRAM STAIN.										
	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms	Type.	Bifidus.	Acidophilus.	Large subtiloid.	Small subtiloid.	Spores.	Coli.	Cocci.	Long chains.	Short chains.	Spiral forms.	Type.
VII-19...	|	+	+	—	+	|	—	—	—	+	+	|	+	—	|	—	—	—	—	—	+	+
20...	?	+	|	—	+	—	—	—	—	+	+	|	+	—	|	—	—	—	—	—	+	+
21...	—	|	+	+	|	+	|	—	—	+	±	+	+	++	+	—	+	+	—	—	++	±
22...	—	—	+	+	|	+	|	—	—	+	±	—	?	++	+	—	+	+	—	—	+	±
23...	—	|	+	+	|	+	|	—	—	+	±	—	?	++	+	—	++	+	—	—	+	±
24...	—	—	++	+	+	+	|	—	—	+	±	—	—	+++	+	—	+	—	—	—	+	±
25...	—	—	+	+	—	+	|	—	—	+	±	—	—	+++	+	—	+	—	—	—	+	±
26...	—	—	+	+	—	+	|	—	—	+	±	—	—	+++	+	—	+	—	—	—	+	±
27...	—	|	+	+	—	+	+	—	—	+	±	—	—	+++	+	|	+	—		—	+	±
28...	—	|	+	+	—	+	|	—	|	+	±	—	—	+++	+	—	+	—	—	—	+	±
29...	—	|	+	+	—	+	—	—	|	+	±	—	|	+++	++	—	++	—	—	—	+	±
30...	—	?	++	++	—	+	—	—	—	+	±	+	+	+++	++	—	++	+	—	—	++	±
31...	—	|	++	+	—	+	—	—	—	+	±	+	+	++	++	—	+	+	—	—	++	+
VIII-1...	—	|	++	+	—	+	—	—	—	+	±	+	+	++	++	—	+	+	—	—	++	+
2...	—	|	++	+	—	+	—	—	—	+	±	—	+	++	++	—	+	|	—	—	++	±
3...	—	+	+	|	—	+	+	—	—	+	+	—	+	++	+	—	|	|	—	—	+	+
4...	—	+	|	—	|	—	—	—	—	+	+	—	+	++	++	—	|	|	—	—	+	+
5..	—	+	+	+	—	—	+	—	—	+	+	++	+	++	++	—	|	|	—	—	+	+
6...	+	+	|	|	|	|	+	—	—	+	+	++	+	++	+	—	+	|	—	—	+	+
7...	—	+	+	|	—	|	+	—	—	|	+	++	+	+	++	+	—	+	—	|	++	+
8...	+	+	+	+	—	|	+	—	—	+	+	—	+	++	+	—	+	|	|	—	++	+
9...	—	+	+	—	+	|	—	—	—	+	+	+	+	++	+	—	+	+	—	—	++	+
10...	—	+	+	—	+	—	—	—	—	+	+	—	+	+	|	—	+	|	—	—	+	+
11...	—	+	+	+	|	+	|	—	—	|	+	—	+	+	+	—	+	|	—	—	+	+
12...	—	+	+	+	—	+	|	—	—	+	+	—	|	+	+	—	+	—	—	—	+	±
13...	—	|	+	+	—	+	|	—	—	+	±	—	|	++	++	—	++	—	—	—	|	±
14...	—	|	++	+	—	+	|	—	—	—	±	+	+	+++	++	—	++	|	—	—	+	±
15...	—	+	++	+	|	+	—	—	—	+	±	—	+	+++	++	—	++	|	+	—	+	±
16...	—	|	++	+	|	+	—	—	—	+	±	—	|	++	++	+	++	|	+	—	+	±
17...	—	|	++	+	|	+	|	—	|	+	±	—	—	++	++	—	++	+	—	—	—	±
18...	—	|	++	+	—	++	+	+	|	+	±	—	|	++	+	—	+	|	—	—	+	±
19...	—	|	++	+	—	++	+	—	|	+	±	—	+	+	|	—	+	|	—	—	++	+
20...	—	+	|	|	—	+	|	—	—	+	±	+	++	?	?	—	—	|	+	—	++	+
21...	—	+	|	|	—	?	|	—	—	++	+	+	++	|	?	—	—		—	—	++	+
22...	—	+	+	|	—	?	+	—	—	+	+	—	++	?	?	—	—	—	—	—	++	+
23...	—	+	|	?	—	?	|	—	—	++	+	—	+	+	|	—	|	—	—	—	++	+
24...	—	+	+	—	—	|	—	—	—	+	+	—	+	+	—	+	|	—	—	—	++	+
25...	—	+	+	|	—	+	—	—	—	+	±	—	+	++	+	—	+	|	—	—	+	±
26...	—	|	+	+	—	+	—	—	—	|	±	—	?	++	++	—	+	—	—	—	+	±
27...	—	|	+	+	—	+	|	—	—	|	±	—	?	++	++	—	+	—	—	—	|	±
28...	—	|	+	+	—	+	—	—	—	|	±	—	—	++	++	—	++	—	—	—	|	±
29...	—	?	++	+	—	++	+	—	—	|	±	—	—	++	++	—	++	—	—	—	|	±
30...	—	?	++	+	—	++	+	—	—	|	±	—	—	++	++	—	++	—	—	—	|	±

TABLE VII.—Continued.

Fermentation tubes.				Urine.			Milk.			Acidophiles acetic acid broth.			Diet.	Remarks.
Dextrose.	Lactose.	Saccharose.	Type of growth.	Indican.	Indolacetic acid.	Millon's.	Coagulated.	Gas.	Peptonized.	5 per cent.	10 per cent.	20 per cent.		
7	7	7	+											
5	5	6	+				++	|	+	++	++	+	Eggs	
30	55	60	++	++	−	++	++	90	+	++	++	+	"	
40	75	80	++	++	−	++				++	|	|	"	
35	55	80	++	++	−	++							"	
80	75	80	++	++	−	++				++	−	−	"	
75	75	75	++	+	−	+							"	
65	85	65	++	++	−	++	+	100		+	−	−	"	
70	80	65	++	+	−	++	+	100	+				"	
90	85	80	++	+	−	+							"	
75	75	70	++	+	−	+	+	40	+				"	
25	20	15	++	|	−	|	+	20	+				"	
15	10	20	+										"	
15	10	10	+										Milk and dextrose	
5	3	5	+										"	
2	3	2	+	−	−	−							"	
2	0	2	+	−	−	−							"	
0	2	3	+	−	−	−							"	
0	0	2	+										"	
5	2	4	+	−	−	−							"	
5	7	1	+	−	−	−	−						"	
2	2	2	+										"	
1	5	1	+										"	
1	1	2	+										"	
15	15	30	+										Eggs	
45	55	60	++	+	−	+							"	
25	25	30	++										"	
55	55	65	++	+	−	++							"	
80	80	75	++										"	
70	70	70	++	+	−	+							"	
40	30	40	++	+	−	+							"	
20	20	15	++										"	
1	2	5	+	+	−	+							"	
3	2	2	+										"	
7	2	3	+	|	−	+							"	
1	4	1	+	−	−	−							"	
1	3	2	+	|	−	|							"	
15	20	15	++										"	
85	70	75	++	+	−	++							"	
65	60	70	++										"	
50	45	50	++										"	
75	70	65	++	++	−	++							"	
60	70	65	++										"	

EXPLANATION OF THE PLATES.

PLATE I. Fig. I. Protein diet. Monkey. Feces. The Gram-stained fields show mixed Gram-positive and Gram-negative flora. The former consists to a considerable degree of large and small subtiloid bacilli, with a few coccal forms. The latter is composed largely of organisms referable morphologically and culturally to *B. coli* and its variants. *1* and *2*, large subtiloid organisms; *3*, *B. coli*; *4*, small subtiloid bacilli.

FIG. II. Protein diet. Monkey. Dextrose sediment. The morphological differences are brought out more clearly, due in all probability to the fact that the organisms are in active vegetative development. *1* and *2*, large subtiloid bacilli; *3*, *B. coli*; *4*, small subtiloid bacilli.

PLATE II. Fig. III. Carbohydrate diet. Monkey. Feces. The fields differ from those of the protein diet, being much more homogeneous, both with respect to the staining and to the morphology of the organisms. The most prominent type represented is *B. acidophilus*, with, however, a moderate number of *B. bifidus*. It is not possible to differentiate with certainty between these two organisms, morphologically, unless one sees them in artificial media.

Fig. IV. Carbohydrate diet. Monkey. Dextrose sediment. This sediment is less characteristic than many derived from this source, but is introduced to show the morphology of those organisms characteristic of this diet. *1*. *B. acidophilus*, long curved form; *2*, *B. acidophilus*, shorter form (this organism is more slender than the large subtiloid bacillus, and longer than a small subtiloid organism); *3*, curved form of *B. acidophilus*; *4*, typical *B. bifidus*.

PLATE III. Fig. V. Transitional stage from carbohydrate to protein diet. Monkey. Feces. The organisms, with the exception of the large forms, are undergoing a granular degeneration. This is characteristic of the appearance of the Gram-stained feces during that period elapsing from the disappearance of the carbohydrate flora to the establishment of the protein flora. The organisms diminish in size, and stain irregularly. *1*, degenerating acidophiles; *2*, subtiloid bacillus.

Fig. VI. Transitional stage. Dextrose sediment. Monkey. The degenerative character of the acidophiles is shown. The organisms develop slowly, and are atypical morphologically and in staining reaction. *1* and *2*, *B. bifidus*; *3*, subtiloid bacilli; *4*, degenerating acidophilic bacteria.

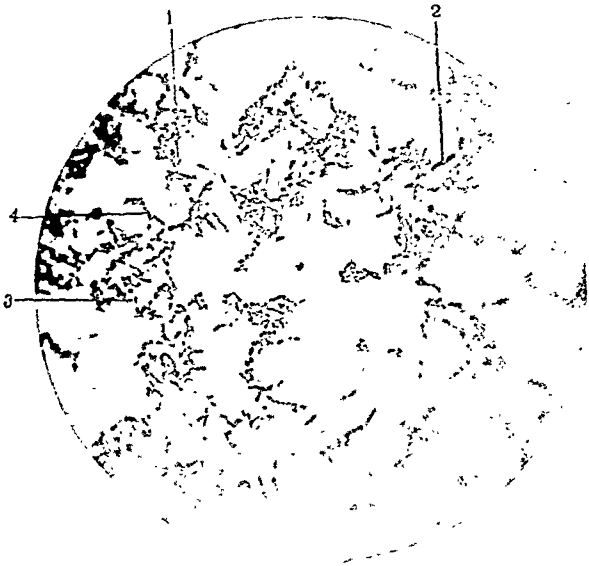

FIG. 1. PROTEIN DIET: FECES. GRAM STAIN.

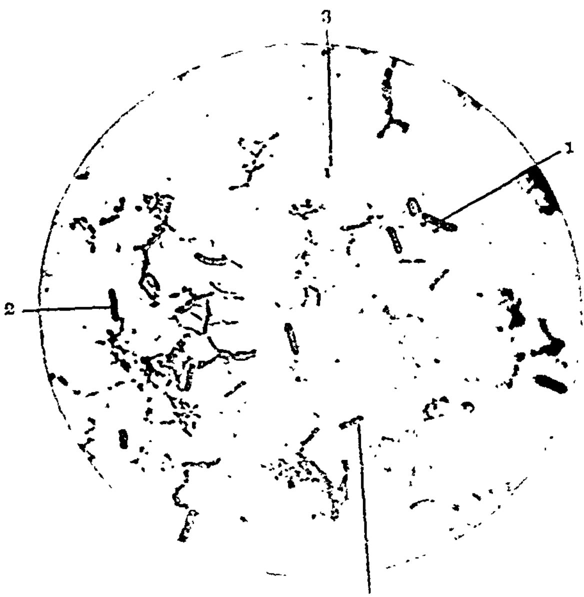

FIG. 2. PROTEIN DIET. DEXTROSE SEDIMENT. GRAM STAIN.

FIG. 3. CARBOHYDRATE DIET: FECES. GRAM STAIN.

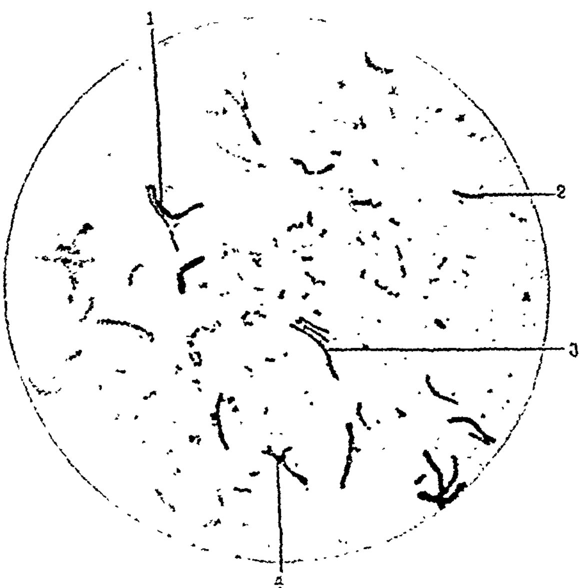

FIG. 4. CARBOHYDRATE DIET. DEXTROSE SEDIMENT. GRAM STAIN.

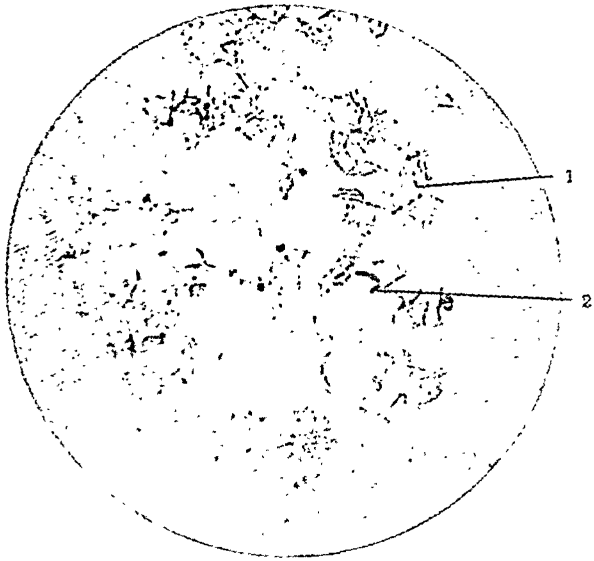

FIG. 5. TRANSITIONAL STAGE: CARBOHYDRATE TO PROTEIN. FECES. GRAM STAIN.

FIG. 6. TRANSITIONAL STAGE: CARBOHYDRATE TO PROTEIN DIET. DEXTROSE SEDIMENT. GRAM STAIN.

THE PURIN FERMENTS OF THE RAT.

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The transformations which purin derivatives undergo in the presence of aqueous extracts of animal tissues can be explained only by assuming the existence in these tissue extracts of at least four active agents: viz., guanase, adenase, xanthoöxidase and uricolase. For a number of reasons these active agents have been termed ferments. They are all thermolabile, all exert their greatest activity near the body temperature and one has been shown to obey the law of velocity required for a catalytic agent.[1] We therefore propose to regard them as ferments. Just what constitutes a proof of the presence or absence of a ferment is a matter that may give rise to some dispute. The presence of adenase in an organ extract would be ideally shown if it were found that in the presence of the extract a given amount of adenin completely disappears and an equivalent amount of hypoxanthin appears in its place. But such an experiment would be almost impossible to execute. With the analytical methods at our disposal, a small amount of material is sure to be lost and, what is a more serious obstacle, the precursors of these substances exist in the tissues themselves, so that there is some danger of a confusion of the introduced base with bases having another origin at least so far as quantitative results are concerned.

We hold the view that the presence of a small amount of the altered product, which does not increase as digestion proceeds, is scarcely any reason at all for assuming the presence of a ferment. A ferment should not be stated when after a greatly prolonged digestion, a reasonable proportion of the initial base can be re-

[1] Burian: *Zeitschr. f. physiol. Chem.*, xliii, p. 497.

covered unchanged. "A trace of a ferment" is a misnomer: we know nothing about the masses of ferments, and a small amount of a ferment means a small amount of time. When the time of digestion has been indefinitely prolonged and the initial material has been recovered in reasonable amount, surely the recovery of a trace of the alteration product is no reason for assuming a trace of the ferment.

On the other hand, the complete absence of a ferment can never be proven; for no matter how long a digestion is allowed to proceed without giving evidence of ferment action, a longer time may be demanded. If it be true that the active agents under discussion are not catalytic agents, then, being incapable of repeating their work, they would manifest themselves according to their mass and produce a partial decomposition which would not increase with the time. Such an assumption though highly improbable would at least explain what have been assumed as differences between dead and living tissue so far as concerns transformations of purin derivatives. The activity of the dead tissue would depend upon the mass of active agent present at death: but in the living organ this may be continually renewed. Such an assumption serves little purpose and is not admissible if the active agents are ferments.

While such considerations suggest that obstacles exist in the way of conclusive results in this field of work, experimenters have usually found this not to be the case. Experience has shown that the initial substance is recovered in reasonable amount without the appearance of the alteration product, or that the initial substance completely disappears with the appearance of a reasonable equivalent of the alteration product. In an examination of muscle, of course, due consideration is given to the fact that this tissue initially contains a relatively large amount of hypoxanthin not present in aqueous extracts of other tissues.

A study of the purin ferments has been of interest from two different points of view. In the first place because by their action, uric acid is produced from substances which are available in the organism. It becomes therefore of the greatest importance to know whether these functions are actually exerted in the living body, or confined to organ extracts, and from this point of view it is desirable that a concordance be shown between the localiza-

tion of the ferments and the chemical composition of the execretions. All animal species thus far examined have been found possessed of the ferments which can account for the formation of uric acid. In this connection it is interesting to note that human urine contains adenin, and that the results of various experimenters are rapidly converging toward the conclusion that human organ extracts do not exhibit adenase.[1]

The study of the purin ferments has been of great interest for another reason. A marked variation in their localization can be shown with variation of organ, animal species and embryonic development so that an animal is characterized by the distribution of the ferments exhibited by aqueous extracts of its several organs.[2] Thus the liver and spleen of the pig do not contain guanase but do contain adenase, while the corresponding organs of the ox show both ferments. The liver of man exhibits both guanase and xanthoöxidase but not adenase. Adenase makes its appearance in the liver of the pig embryo at an earlier stage of development than xanthoöxidase.[3] Whether or not these results are expressions of what is actually going on in living animal organisms and whether or not the differences are qualitative are questions not to the point. The differences are so clear as to be unmistakable and they are in some way expressions of life processes.

The following characteristic distribution of purin ferments has been found in man.

1. No ferment exercising the function adenase can be shown in extracts of any organ thus far examined (liver, spleen, pancreas, muscle, lung, kidney).

2. Xanthoöxidase is actively exerted by the aqueous extract of the liver, but not any other organ.

3. Guanase is found especially in the liver; it can be shown also in a number of other organs but not in muscle.

4. Uricolase which is present in the organs of animals, is not to be found in the organs of man.

[1] Jones and Miller: *Zeitschr. f. physiol. Chem.*, lxi, p. 395; Schittenhelm: *ibid.*, lxiii, p. 248.

[2] Jones: *ibid.*, xlv, p. 84.

[3] Jones and Austrian: this *Journal*, iii, p. 227; Mendel and Mitchell: *Amer. Journ. of Physiol.*, xx, p. 97.

As far as examinations have been made, the distribution of purin ferments in man is not disturbed to any notable extent by disease. Results obtained were found the same in typhoid fever, gout, chronic nephritis and aneurism. Owing to the difficulty of controlling conditions of obtaining material for examination in the case of man it seemed desirable to undertake an examination of animals in disease. For this purpose rats suffering from experimental nagana (trypanosomiasis) were studied and compared with normal animals. No differences could be observed but the normal distribution of purin ferments in the rat was found so peculiar as to deserve special attention.

A few preliminary experiments indicated that guanase is uniformly distributed among all the organs, its presence having been easily demonstrated in the spleen, kidney, liver and muscle. On the other hand a large proportion of introduced adenin could be recovered unchanged after digestion at 40° with aqueous extracts of the same organs. While no special effort was made to demonstrate xanthoöxidase, the experiments with guanin made it appear probable that the ferment was not present, for uric acid could not be detected among the products.

A more careful series of experiments was then undertaken and as the absence of two ferments was to be expected, a departure from the ordinary method of analysis was adopted, which we had occasion to know would lead to results more nearly quantitative. So far as concerns a possible loss of either guanin or uric acid by the use of this method, it may be stated that the results obtained with its use were checked by the older and more laborious process. The finely ground tissue was treated with four times its weight of water and enough chloroform to prevent putrefaction. After sufficient agitation and standing several hours, the aqueous extract was strained off and treated with an alkaline solution of the purin base. Where the absence of the ferment was suspected, considerably less than 1 milligram of base was used for 1 cc. of organ extract and the subsequent digestion was prolonged; where the presence of the ferment had been indicated, considerably more than 1 milligram of base was used for 1 cc. of organ extract and the time of digestion correspondingly shortened. After digestion at 40°, the product was made faintly acid with acetic acid, heated to boiling and the coagulum filtered off.

While still warm, the filtered fluid was treated with a slight excess of basic lead acetate, the precipitate filtered off, and the excess of the reagent removed from the boiling fluid with sulphuric acid. The filtrate from lead sulphate was evaporated on the water-bath, enough ammonia was added to give a strong alkaline reaction and the bases precipitated with an ammoniacal solution of silver nitrate.

So far as the admissibility of the use of basic lead acetate is concerned, it is certain that no objection can be offered in connection with adenin or hypoxanthin. While the difficult solubility of uric acid, guanin and xanthin might suggest a possible source of error, experiments made with the organs of animals other than the rat, prove the soundness of the method in connection with all three of these substances. Guanin was recovered nearly quantitatively after digestion with an aqueous extract of human muscle and uric acid was isolated from human liver after digestion with guanin. The results described below are a sufficient guarantee for the recovery of xanthin by this method.

The use of basic lead acetate is of the greatest advantage. It removes traces of proteid which escape coagulation by heat so that subsequent boiling with sulphuric acid for this purpose is avoided. This latter procedure always produces brown pigment which is very difficult to remove and whose presence alters considerably the solubility of the bases in the scheme adopted for their separation from one another. Basic lead acetate is also effective in removing substances that tend to increase the solubility of the silver compounds of the purin bases, so that the precipitation with silver nitrate is quantitative and the subsequent decomposition of the silver precipitate is never attended with the production of an emulsion which often results in the abandonment of the experiment. The removal of the phosphates by basic lead acetate is of advantage in connection with the isolation of guanin as it does away with the necessity of separating the guanin and phosphates by treatment with caustic soda and precipitation of the guanin from the alkaline solution with acetic acid.

Aside from the departure noted, the following results were obtained by the methods which are in common use.

GUANASE AND XANTHOÖXIDASE.

1. Aqueous extract of rats muscle .... 260 cc.
   Guanin hydrochlorate in caustic soda .... 262 mg.
   Equivalent guanin .... 175 "
   Digestion at 38° with passage of air 3 days.
   Found: Xanthin 140 mg. (80 per cent of guanin);
   Guanin absent;
   Uric acid not detected;
   Hypoxanthin fraction contained purin material as is always the case with muscle.
2. Aqueous extract of rats muscle .... 275 cc.
   Guanin hydrochlorate in caustic soda .... 420 mg
   Equivalent guanin .... 280 "
   Digestion at 38°, 3 days: frequent agitation with fresh air.
   Found: Xanthin 274 mg. (98 per cent of guanin);
   Guanin absent;
   Hypoxanthin as in exp. 1;
   Uric acid not detected.
3. Aqueous extract of rats liver .... 280 cc.
   Guanin hydrochlorate .... 420 mg.
   Equivalent guanin .... 280 "
   Digestion at 38°, 3 days: frequent agitation with fresh air.
   Found: Xanthin 236 mg. (84 per cent of guanin);
   Guanin absent;
   Uric acid not detected;
   Hypoxanthin not detected.
4. Aqueous extract of kidney .... 50 cc.
   Guanin hydrochlorate .... 75 mg.
   Equivalent guanin .... 50 "
   Digestion at 38°, 3 days: occasional passage of air.
   Found: Xanthin 42 mg. (84 per cent of guanine).
   Guanin absent.
   Uric acid not detected.
   Hypoxanthin not detected.
5. Aqueous extract of spleen .... 30 cc.
   Guanin hydrochlorate .... 40 mg.
   Equivalent guanin .... 27 "
   Digestion at 38° 3 days: occasional passage of air.
   Found: Xanthin 24 mg. (92 per cent of guanin).
   Guanin absent.
   Uric acid not detected.
   Hypoxanthin not detected.

In order to test the activity of guanase in rats muscle, 1000 cc. of the aqueous extract was treated with 2 grams of guanin hydro-

chlorate in the proper amount of alkali, with the intention of removing portions of the material for examination after various increasing intervals of time. The first portion was removed after digestion at 38° had continued for 17 hours, when it was found that the relatively large amount of guanin had been completely changed to xanthin.

6. Aqueous extract of muscle..............................250 cc.
   Guanin hydrochlorate..............................500 mg.
   Equivalent guanin..............................333 "
   Digestion at 38° for 17 hours.
   Found: Xanthin 300 mg. (90 per cent of guanin);
   Guanin absent.

The xanthin obtained in a number of experiments with various organs of the rat was thoroughly mixed by grinding. One gram of the material was examined for uric acid by Horbaczewski's method. Uric acid could not be detected. The xanthin was recovered, purified through the nitrate and analysed.

I. 0.1648 gm. of material required; 18.4 cc. standard acid (1 cc-.003294).
II. 0.1134 gm. of material required; 12.5 cc. standard acid (1 cc. = .003294).

	Theoretical for xanthine,	Found I	Found II
N..............................	36.8	36.7	36.4

A number of experiments were made with extracts of the organs of the rat to specially test the extracts for xanthoöxidase. Air was continually passed during the digestions and although convinced that the method of isolation was not responsible for any loss of uric acid, we used the older method. Our results without exception lead to the conclusion that while *guanase is distributed uniformly and in great activity among all the organs of the rat, xanthoöxidase is present in none of them.*

ADENASE.

The following are the results obtained by digesting adenin with aqueous extracts of the organs of the rat. The methods employed were the same as those in common use with the departure already noted. As the absence of adenase had been indicated, relatively small quantities of adenin were used.

1. Aqueous extract of rats liver........................170 cc.
   Adenin sulphate in alkali........................200 mg.
   Equivalent adenin........................133 mg.
   Digestion at 38°, 6 days.
   Found: Adenin picrate 380 mg. (recovery quantitative); Hypoxanthin absent.
2. Aqueous extract of rats liver........................150 cc.
   Adenin sulphate in alkali........................86 mg.
   Equivalent adenin........................57 "
   Digestion at 38°, 6 days.
   Found: Adenin picrate 140 mg. (recovery 91 per cent); Hypoxanthin absent.
3. Aqueous extract of rats muscle........................250 cc.
   Adenin sulphate in alkali........................86 mg.
   Equivalent adenin........................57 "
   Digestion at 38°, 6 days.
   Found: Adenin picrate 148 mg. (recovery 96 per cent). Hypoxanthin present as is always the case with muscle.
4. Aqueous extract of kidney........................50 cc.
   Adenin sulphate in alkali........................65 mg.
   Equivalent adenin........................43 "
   Digestion at 38° 6 days.
   Found: Adenin picrate 120 mg. (recovery quantitative); hypoxanthin absent.

The smaller organs (spleen and lung) did not afford enough material for a quantitative examination but adenin could be recovered as picrate after digestion with aqueous extracts of these organs.

The various specimens of recovered adenin picrate were found to melt around 280°. Upon recrystallization from hot water they formed characteristic clusters of fine needles, leaving a mother liquor which gave no indication of the presence of any purin base (hypoxanthin) when treated with ammonia and silver nitrate.

A quantity of adenin picrate obtained after digestion with extracts of rats organs was converted into the sulphate and analysed.

Weight of picrate, 1.32 gm. (equivalent to 488 mg. of base).
Weight of sulphate, 0.65 gm. (equivalent to 433 mg. of base).
0.1432 gm. of substance required 14.9 cc. of standard acid (1 cc. = .0033 gram N).

	Theoretical	Found
N........................	34.6	34.3

These experiments show that *no ferment with the function adenase is present in the organs of the rat.*

Experiments with the Combined Organs of the Rat.

The absence of both adenase and xanthoöxidase from the organs of this animal appeared curious. Although adenase is not present in extracts of human organs, yet every animal species including man has been found to possess in one organ or another the ferments necessary for the formation of uric acid from guanin, adenin or both. The following experiments were, therefore, undertaken in order to find whether one or both of the ferments missing in the organ extracts of the rat might not be found in some of the smaller organs that had not been examined, or whether a precursor of the ferments present in one organ might require an activator located in another organ.

An aqueous extract was made of the combined organs of the entire animal and closely examined for the two ferments in question with the following results:

1. Aqueous extract of entire rat....................1000 cc.
   Adenin sulphate in alkali....................516 mg.
   Equivalent adenin....................344 "
   Digestion at 40° for 6 days.
   Found: Adenin picrate 927 mg.
   Equivalent adenin 343 mg. (recovery quantitative).
2. Aqueous extract of entire rat....................400 cc.
   Hypoxanthin in alkali....................300 mg.
   Digestion at 38°, 4 days passage of air.
   Found: Hypoxanthin nitrate 455 mg.
   Equivalent hypoxanthin 303 mg.

The hypoxanthin nitrate consisted uniformly of whetstone crystals and gave no response whatever to the sensitive color reaction for xanthin.

CONCLUSION.

The results of this work show that the organ extracts of the rat jointly and severally are incapable of exhibiting either adenase or xanthoöxidase. There is therefore no way for uric acid to be formed by the purin ferments in extracts of the organs of the animal. Nevertheless rats' urine contains uric acid. From 50 cc. of

urine we were able to isolate enough uric acid for complete identification. Presumably the organs also contain uric acid which might be detected by methods of sufficient refinement but the substance cannot be produced by the action of organ extracts on purine bases.

So far as adenase is concerned we are reminded of a similar condition which has recently come to light in connection with dogs' muscle and which undoubtedly holds for all muscles. This tissue by common consent is either entirely free from adenase or sufficiently poor in this function to attract special notice: but it is not lacking in hypoxanthin. It was dogs' muscle that formed the experimental basis of Burian and Schur's well known discovery that muscular hypoxanthin is dependent upon muscle stimulation.[1] The gist of their entire work is against the assumption that the hypoxanthin of muscle has its origin in the dead leucocytes. Their conclusion is supported by the absence from dogs' muscle extract of adenase, a ferment which forms an indispensable link in the passage from nucleic acid to hypoxanthin. Moreover the hypoxanthin in the muscles of various animal species shows no variation with the adenase which extracts of these muscles exhibit it. Adenase cannot be demonstrated in extracts of the muscles of rabbit, pig, dog and man; it is in great evidence in ox muscle: but the hypoxanthin is practically the same in all.

The hypoxanthin of dogs' muscle and the uric acid of rats' urine are apparent anomalies of the same order; each represents a metabolic product not accounted for by the purin ferments exhibited by aqueous extracts of the tissues. In explanation of such a condition two hypotheses present themselves.

I. The nuclein ferments may be at work in the living organs but fail to exhibit themselves in organ extracts. This assumption is supported by an analogy. The nuclein ferments *do* exhibit themselves in aqueous organ extracts: they were discovered in this connection.

II. Hypoxanthin and uric acid may be formed in the living organism by processes which do not involve the purin ferments. So far as this hypothesis concerns uric acid the recent work of Ascoli and his co-workers leaves no doubt of its truth.

[1] *Zeitschr. f. physiol. Chem.*, xliii, p. 533.

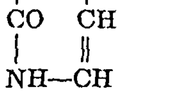
The results of numerous investigations have shown that when an extract of certain animal organs are digested with uric acid and air passed into the digesting mixture, the uric acid is decomposed. The principal decomposition product has been shown to be allantoïn. Ascoli and Tzar[1] found that if the digestion of the decomposition products be continued with passage of carbon dioxide, uric acid is regenerated. This regeneration of uric acid can be accomplished under conditions which with passage of air do not produce a decomposition of uric acid. The process is therefore not simply a reversal of ferment action but involves two different ferments. The destroying ferment is present in the aqueous extract of the blood-free liver of the dog, and its action is ended by boiling the aqueous extract. The regenerating ferment is present in the blood, not in the organ extract. The latter contains only an activator which is not affected by boiling the aqueous extract. Finally Ascoli and Tzar[2] succeed in producing uric acid by treating an aqueous extract of liver with blood that contains dialuric acid and urea. We find here a synthesis of uric acid under the influence of organ extracts, which involves neither a purin ferment nor a purin ring.

[1] *Zeitschr. f. physiol. Chem.*, lviii.
[2] *Zeitschr. f. physiol. Chem.*, lxii.

This, he stated, crystallized from water in small prismatic needles. His analysis gave it the formula, $C_5H_5N_2O_2K+H_2O$. The alkali salts of thymine and uracil appear to have received no further attention until very recently, when the mono-potassium salts of both these compounds were described by Johnson and Clapp.[1] The potassium salt of uracil was found to contain one molecule of water of crystallization, but in the case of the potassium salt of thymine the water of crystallization was not determined. In 1885 in his investigations on 4-methyluracil, Behrend[2] prepared both the sodium and potassium salts of this pyrimidine. Both were found to contain one molecule of water of crystallization and were similar in properties to the salts of thymine and uracil just mentioned.

The cytosine, thymine and uracil employed in the experiments here described and in the physiological experiments previously referred to, were synthesized according to the methods described by Prof. H. L. Wheeler and his collaborators, Prof. T. B. Johnson and others. The ease with which the syntheses may be carried out and the good yields obtained by one inexperienced with the methods indicate their value. The preparations of these pyrimidines described in this paper were the ones employed in the above mentioned physiological experiments.

Cytosine.—Cytosine was prepared according to the directions given by Wheeler and Johnson[3], which is the only synthesis of this compound described. As the basis for the various calculations of this synthesis 150 grams of ethylacetate were used.



Cytosine

The total yield of cytosine in the form of the hydrochloride was 15.5 grams. This compound is extremely soluble in water and on this account especially suited to physiological work. The

[1] Johnson and Clapp: This *Journal*, v, p. 49, 1908.
[2] Behrend: *Annalen der Chemie*, ccxxix, p. 1, 1885.
[3] *Loc. cit.*

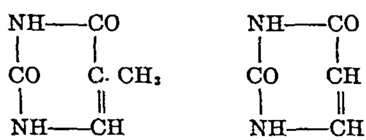
hydrochloride, $C_4H_5N_3O \cdot HCl \cdot H_2O$ quickly loses its molecule of water of crystallization on exposure to the air. When a sample of this preparation was dried at 140 C., a nitrogen determination (Kjeldahl) resulted as follows:

	Calculated for $C_4H_5N_3O \cdot HCl$:	Found:
N....................................	28.65	28.31

Nitrogen determinations on the picrate in all cases gave nearly theoretical results with the salicylic acid modification of the Kjeldahl-Gunning method, and that too, after recovery from the animal organism.

	Calculated for	Found:			
	$C_4H_5N_3O \cdot C_6H_2(NO_2)_3OH$:	I.	II.	III.	IV.
N...............	24.63	24.78	24.67	24.68	24.70

Below are described several new salts of thymine and uracil, together with the potassium salts of these two compounds which have previously been described. The exact constitution of these salts is not known but it seems probable that the alkali metal would be joined in the most negative position, namely to the oxygen atom or atoms.



Thymine Uracil

THYMINE.—The thymine employed for the preparation of the various salts was synthesized according to the methods described by Wheeler and Merriam and Wheeler and Johnson.[1] Starting with 115 grams of ethyl propionate as the basis of the various calculations for the condensations, 27.5 grams of pure thymine were obtained. It consisted of large lustrous perfectly white plates. Analysis (Kjeldahl):

	Calculated for $C_5H_6N_2O_2$:	Found:
N....................................	22.23	22.09

[1] Wheeler and Merriam: *American Chemical Journal*, xxix, p. 478, 1903. Wheeler and Johnson: *Ibid.*, xxxi, p. 591, 1904.

Sodium salt of thymine, $C_5H_5N_2O_2Na$. One molecular proportion of sodium hydroxide was dissolved in 15 cc. of warm water and 2.5 grams of thymine added. The thymine quickly went into solution and alcohol was added almost to the point of crystallization. The salt separated in long needles, some of which were a centimeter in length. About 1.0 gram of the salt was obtained from the first crystallization. It seems to be considerably more soluble than the corresponding salt of uracil and apparently contains no water of crystallization. Analysis (Kjeldahl):

I. First preparation dried over sulphuric acid.
II. Second preparation dried in air.
III. Second preparation dried at 170 C.

	Calculated for $C_5H_5N_2O_2Na+H_2O$:	Calculated for $C_5H_5N_2O_2Na$:	Found: I.	II.	III.
N..........	16.87	18.93	18.67	18.48	18.84

Potassium salt of thymine, $C_5H_5N_2O_2K+\frac{1}{2}H_2O$. One-half gram of thymine was dissolved in 5 cc. of a solution containing one molecule of potassium hydroxide and a small amount of alcohol added. The potassium salt separated in balls of little needles. It was filtered off, washed with alcohol and dried over sulphuric acid. According to the analyses given below this salt contains one-half molecule of water of crystallization. Analysis (Kjeldahl):

Salt dried over sulphuric acid.

	Calculated for $C_5H_5N_2O_2K+\frac{1}{2}H_2O$:	Found:
N..................................	17.07	17.18

Salt dried at 170° C.

	Calculated for $C_5H_5N_2O_2K$:	Found:
N..................................	18.48	18.28

The water of crystallization was determined in a second preparation by heating at 170° C. 0.2841 gram of substance lost 0.0174 gram H_2O.

	Calculated for $C_5H_5N_2O_2K+\frac{1}{2}H_2O$:	Found:
H_2O............................	5.80	6.18

Mercury salt of thymine, $C_5H_4N_2O_2Hg$. One-half gram of thymine was treated with exactly one molecule of alkali in a small amount of water and a solution of mercuric chloride added

in slight excess. A white precipitate quickly settled out in a somewhat gelatinous condition; and it was thoroughly washed with water and alcohol. Analysis (Kjeldahl) after drying over sulphuric acid:

	Calculated for $C_5H_4N_2O_2Hg$:	Found:
N....................................	8.65	8.74

Lead salt of thymine, $C_5H_4N_2O_2Pb+2H_2O$. One-half gram of thymine was dissolved in a molecular proportion of sodium hydroxide and then an excess of lead acetate solution added. A crystalline precipitate settled out at once, which was composed of little leaves. A nitrogen determination (Kjeldahl) gave 18.65 per cent, showing that it was in all probability impure thymine. From this filtrate, bundles of short needles gradually crystallized out after several days. The volume of the solution was about 100 cc. The salt was filtered off, washed with water, then with alcohol and dried over sulphuric acid. It contains two molecules of water of crystallization. Analysis (Kjeldahl):

Salt dried over sulphuric acid.

	Calculated for $C_5H_4N_2O_2Pb+2H_2O$:	Found:
N................................	7.64	7.40

Salt dried at 170° C.

	Calculated for $C_5H_4N_2O_2Pb$:	Found:
N................................	8.47	8.13

When 0.2206 gram of the salt was dried at 170° C. 0.0204 gram of H_2O was lost.

	Calculated for $C_5H_4N_2O_2Pb+2H_2O$:	Found:
H_2O................................	9.35	9.25

Uracil.—The uracil employed for the preparation of the various salts described below was synthesized according to the method described by Wheeler and Liddle.[1] Starting with 150 grams of ethylacetate as the basis of the various calculations

[1] Wheeler and Liddle: *American Chemical Journal*, xl, p. 547, 1908.

for the synthesis of uracil, 42 grams were obtained. The uracil separated from hot water on cooling in the form of little white burrs. Analysis (Kjeldahl):

	Calculated for $C_4H_4N_2O_2$:	Found:
N..................................	25.03	25.25

Sodium salt of uracil, $C_4H_3N_2O_2Na + \frac{1}{2} H_2O$. One gram of uracil was added to 5 cc. of a hot aqueous solution containing 0.6 gram of sodium hydroxide. It dissolved very readily. To this solution was added about 1 cc. of alcohol and the solution warmed. On cooling, the sodium salt of uracil crystallized in little balls of poorly formed needles, which were not distinctly crystalline. The yield was 1.8 grams. This salt has one-half molecule of water of crystallization. Analysis (Kjeldahl):

I. Dried for several days over sulphuric acid.
II. Dried at 118° C.

	Calculated for $C_4H_3N_2O_2Na + \frac{1}{2}H_2O$:	Found: I.	Found: II.
N........................	19.59	19.65	19.63

I. Dried at 140° C. for several days.
II. Dried for one hour at 170° C.

	Calculated for $C_4H_3N_2O_2Na$:	Found: I.	Found: II.
N..........................	20.90	20.12	20.83

A second preparation of this salt was made in the same manner. A nitrogen determination (Kjeldahl) after drying over sulphuric acid resulted as follows:

	Calculated for $C_4H_3N_2O_2Na + \frac{1}{2}H_2O$:	Found:
N..............................	19.59	19.66

Potassium salt of uracil, $C_4H_3N_2O_2K + H_2O$. For its preparation, 3.0 grams of uracil and 2.2 grams (1.5 mol) of potassium hydroxide were employed. They were dissolved in water and crystallized from alcohol. The yield was 3.8 grams. The salt crystallized in long needles and balls of needles. It has one molecule of

water crystallization as has been shown by Johnson and Clapp.[1] Analysis (Kjeldahl) after drying over sulphuric acid:

	Calculated for $C_4H_3N_2O_2K+H_2O$:	Found:
N	16.66	16.98

I. Dried at 118° C.
II. Dried at 140° C.

	Calculated for $C_4H_3N_2O_2K$	Found: I.	Found: II.
N	18.65	18.40	18.76

Mercury salt of uracil, $C_4H_2N_2O_2Hg$. Two grams of uracil were dissolved in an aqueous solution of the molecular proportion of potassium hydroxide and an excess of mercuric chloride added. A flocculent precipitate settled out which was filtered, washed with alcohol and dried to constant weight. The salt was amorphous and weighed 2.2 grams. After filtration, it was noted that more of the salt settled out, indicating that it was not entirely insoluble in water and that alcohol depressed the solubility. Analysis (Kjeldahl):

	Calculated for $C_4H_2N_2O_2Hg$:	Found:
N	9.04	8.94

The solubility of the mercury salt was tested in the following way. One-half gram of the potassium salt of uracil was dissolved in about 15 cc. of water and two gram molecules of the yellow oxide of mercury added and the solution heated. A jelly-like mass separated out. The filtrate from this, when disintegrated with hydrogen sulphide, gave a strong reaction for uracil with bromine-water and baryta-water.[2]

The mercury salt may partially dissociate as the following experiment shows. One-half gram of the mercury salt of uracil was boiled with 50 cc. of water for 5 minutes, allowed to stand over night and filtered off. Of the 0.5 gram 0.44 gram still remained undissolved, and on evaporating the filtrate to dryness 0.07 gram were recovered, making a total of 0.51 gram. The

[1] *Loc. cit.*
[2] Cf. Wheeler and Johnson: This *Journal*, iii, p. 183, 1907.

salt which remained undissolved was subjected to a nitrogen determination (Kjeldahl) with the following result:

	Calculated for $C_4H_2N_2O_2Hg$:	Found:
N....................................	9.04	7.96

This result indicates a partial dissociation into mercuric hydroxide and uracil.

Lead salt of uracil, $C_4H_2N_2O_2Pb$. By precipitating a solution of the potassium salt of uracil with a molecular proportion of lead acetate, an amorphous, snow white salt was obtained. The yield indicated that it was much more soluble than the mercury salt. Analysis (Kjeldahl) after drying over sulphuric acid:

	Calculated for $C_4H_2N_2O_2Pb$:	Found:
N....................................	8.84	8.76

Both thymine and uracil form gelatinous salts with silver when prepared by adding a solution of silver nitrate to a solution of the alkali salts. On account of the very gelatinous nature of these salts it is very difficult to purify them for analysis. Behrend[1] noted this same property with 4-methyluracil. The salts of thymine are all more soluble than the salts of uracil. With uracil it was possible to obtain a quantitative yield of the potassium and sodium salts at the first crystallization, but this was never the case with thymine. In the case of thymine the sodium salt has a greater tendency to crystallize than the potassium salt, while with uracil the reverse is true. There is a tendency on the part of all these salts to partially dissociate and on this account the alkali salts of uracil and thymine are much more soluble in a slight excess of the alkali. Attempts were made to prepare the copper salts of both thymine and uracil. In the case of thymine the attempt met with entirely negative results. With uracil a copper salt was apparently formed, but it dissociated so quickly that at best only a mixture of copper hydroxide and uracil could be obtained. Behrend obtained somewhat similar results with 4-methyluracil. He prepared the copper salt, but stated that on washing with water, the 4-methyluracil was removed

[1] *Loc. cit.*

and copper hydroxide remained. In the case of uracil, attempts to prepare pure or definite salts with a large number of other metals were all unsuccessful.

The fact that one molecule of water of crystallization was found to be present in the case of the potassium salt of uracil and the high temperature required to remove this, suggests the open chain compound $NH_2 \cdot CO \cdot NH \cdot CH{:}CH \cdot COOK$. However, in view of the results obtained with other salt, which apparently contain only a half molecule of water or none at all, the validity of this idea is questioned.

Many physiological chemists refer to thymine, cytosine and uracil as the pyrimidine "bases." The salts of thymine and uracil which have been described, clearly demonstrate the acid properties of these two compounds. In view of this fact, it is hardly correct to refer to these three pyrimidines collectively as "bases."

The sodium salts of thymine and uracil, described above, are obviously the only soluble compounds of these two pyrimidines which can advantageously be used for physiological work. By means of their sodium salts both thymine and uracil can be administered, not only in soluble form, but also in considerable concentration. The disadvantage of these salts is their alkaline reaction, due to the partial dissociation of the salts with the liberation of free alkali.

Aside from the Kossel-Jones method of precipitating the pyrimidines with silver nitrate and baryta-water, the only method which the analytical data given above suggests, is precipitation with mercury. From the general behavior of these pyrimidines, complete precipitation should result, if mercuric sulphate were added in excess to a solution containing them, and then the reaction rendered alkaline with sodium or potassium hydroxide, thus insuring conversion into the insoluble mercury salt. Cytosine is also precipitated by mercuric sulphate in this way.

Uracil appeared to be completely precipitated by Hopkins' reagent (mercuric sulphate in acid solution) but in the case of thymine the precipitation did not appear to be complete unless alkali was added.

The fact that stable copper salts could not be produced with thymine and uracil indicates that these pyrimidines would not be

precipitated along with the purines by the Krüger-Schmid process. As a matter of experiment it was found that the presence of these pyrimidines in urine did not interfere with the determination of the purines according to the above method.

The writer is under obligation to both Professors H. L. Wheeler and T. B. Johnson for their many suggestions and their instruction in the manipulation of the various syntheses.

SUMMARY.

The preparation of the sodium, mercury and lead salts of thymine and uracil, together with the previously known potassium salts, is described.

The ability to prepare salts of both thymine and uracil with various metals clearly demonstrates their acid properties. On this account it is incorrect to refer to the three pyrimidines obtained from nucleic acids collectively as the pyrimidine "bases."

The sodium salts of both thymine and uracil are convenient for physiological work on account of their solubility. Their disadvantage is the alkaline reaction in aqueous solution due to partial dissociation.

The comparative insolubility of the mercury salts of both thymine and uracil, especially in alkaline solution, suggests this as a method of precipitating these compounds. It is more economical than the Kossel-Jones method and is convenient as a second method of precipitation. Cytosine is also precipitated in this way.

THE PRESENCE OF IODINE IN THE HUMAN PITUITARY GLAND.

By H. GIDEON WELLS.

(*From the Department of Pathology, University of Chicago.*)

(Received for publication, February 12, 1910.)

Many different observations have called attention to the possibility of a relationship between the anterior lobe of the hypophysis and the thyroid glands, and this possibility is supported by their embryologic history. Nevertheless, few attempts have been made to ascertain whether the hypophysis contains the most characteristic chemical constituent of the thyroid, iodine. So far as can be found in the literature the only recorded chemical examinations of the hypophysis for iodine are the following:

In 1896 Baumann[1] reported that he had repeatedly examined human pituitaries for iodine, but always with negative results. He does not mention the amount nor the source of his material.

J. Schnitzler[2] examined large quantities of human pituitary tissue obtained in the autopsy room of the Pathological Institute of Vienna, using in one analysis 19 grams, and in another 24 grams of fresh substance, which represents about forty glands in one and fifty glands in the other analysis. Using this large amount of material he found evidence of the presence of iodine in both lots. He states that he communicated with Baumann concerning this disagreement in their results, and that the latter ascribed Schnitzler's positive finding to the large amount of material used.

During the same year (1896) I analyzed fourteen hypophyses obtained from the autopsy room of the Cook County Hospital, the total dry weight of which was 1.225 grams. In this material was found, by Baumann's method, an easily demonstrable trace

[1] Baumann: *Münch. med Wochenschr.*, xliii, p. 311, 1896.
[2] Schnitzler: *Wien. klin. Wochenschr.*, ix, p. 657, 1896.

of iodine, sufficient to give a distinct reaction with chloroform, the amount being estimated colorimetrically as 0.05 milligram of iodine.[1]

As far as I can learn the subject was not taken up again for over ten years, until in 1909 Halliburton[2] reported an analysis of 1.2 grams of dry pituitary substance from twenty-two human glands, and of 1 gram of dried ox pituitary substance. In neither sample could any trace of iodine be obtained by Baumann's method. Shortly after, Simpson and Hunter[3] reported the analyses of the pituitary bodies obtained from sheep which had previously undergone thyroidectomy, with the object of ascertaining if the increase in colloid which occurs in the pituitary after removal of the thyroid represents a true compensatory functionating on the part of this organ. Although they used a particularly delicate method of analysis, devised by Hunter, no evidence of iodine could be found.

Summarizing these reports we find that two examinations of animal pituitary substance for iodine gave negative results. Of five analyses of human hypophysis for iodine three were positive (Schnitzler (two samples), Wells) and two negative (Baumann, Halliburton). While as a usual thing positive results have more value than negative results, yet in this particular instance the converse is probably true, because of the presence of a source of error present in the human material. This consists in the fact that a considerable proportion of hospital patients receive iodine in some form, and such therapeutically administered iodine may be readily found in any and all tissues of the body, so delicate are our analytic methods for iodine in small amounts. None of the articles quoted contains any mention of this source of error. I have no record of the source of the material used in my own experiments thirteen years ago, which will permit me to ascertain whether the patients had received iodine or not. Therefore I have recently secured, through the kindness of Dr. T. H. Boughton, resident pathologist of Cook County Hospital, twenty-five human pituitary glands. Of these three were from patients who had

1 Wells: *Journ. of the Amer. Med. Assoc.*, xxix, p. 1011, 1897.

2 Halliburton, Chandler and Sikes: *Quart. Journ. of Exp. Physiol.*, ii, p. 240, 1909.

3 Simpson and Hunter: *Proc. Soc. Exp. Biol. and Med.*, vii, p. 11, 1909.

received iodides before death, and these three were preserved and analyzed separately. The other twenty-two were from patients who had not received iodine while in the hospital, and they were analyzed together by Baumann's method, no trace of iodine being found. The three glands from patients who had received iodine gave a distinct but barely discernible pink coloration to the chloroform, indicating the presence of a trace of iodine, too small to estimate quantitatively, but presumably not far from 0.02 milligram since this is about the minimum amount that can be detected by this method.

From the results of all analyses of human pituitary substance reported above, it is evident that the presence of iodine as a normal constituent of this organ must be considered as unproved. To decide this point we need analyses of large numbers of glands taken from the bodies of persons who are known not to have received iodine either internally or from surgical dressings for at least some weeks before death. Positive results obtained by Schnitzler and myself may have been due to therapeutic use of iodine, although, until disproved by analysis of more human material, it still remains possible that the hypophysis may at times contain appreciable quantities of iodine independent of its therapeutic administration. It is also desirable to know if the hypophysis may not have a selective affinity for iodine, similar to that of the thyroid, this causing it to be especially abundant in this tissue in patients who are receiving iodine or iodides.

A NOTE ON THE PHYSIOLOGICAL BEHAVIOR OF IMINOALLANTOÏN AND UROXANIC ACID.

BY TADASU SAIKI.

(*From the Sheffield Laboratory of Physiological Chemistry, Yale University.*)

(Received for publication, January 22, 1909.)

IMINOALLANTOÏN. The recent studies of Wiechowski[1] and others have served to emphasize the long established importance of allantoïn as a possible end product of purine metabolism, and its resistance to chemical disintegration in the animal organism. It may be of interest to record a few notes on the physiological behavior of the closely related compound: *iminoallantoïn.*

```
NH – CH – NH – CO – NH2        NH – CH – NH –CO – NH2
|    |                         |    |
CO   |                         CO   |
|    |                         |    |
NH – C = NH                    NH – C = O
(iminoallantoïn)                 (allantoïn)
```

The compound was prepared according to the directions of Denicke.[2] Care is necessary in the manipulation of solutions containing it, owing to the ease with which iminoallantoïn can be converted into allantoïn, e. g., by continued action of boiling water or brief heating with hydrochloric acid. It was dissolved in a little dilute hydrochloric acid at a low temperature and the excess of acid neutralized at once with alkali. Such solutions were introduced parenterally into rabbits. No toxic symptoms were observed with doses of one gram. In one experiment after the intravenous injection of one gram in 100 cc. at the rate of 1.5 cc. per minute, the urine of the following twenty-four

[1] Cf., for example, Wiechowski: *Biochem. Zeitschr.*, xix, p 368, 1909. Schittenhelm: *Zeitschr. f. physiol. Chem.*, lxii, p. 80, 1909.

[2] Denicke: *Ann. d. Chem.*, cccil, p. 270, 1906.

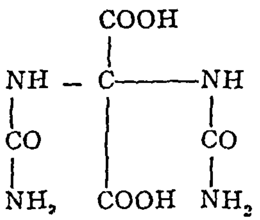
hours yielded 0.607 gram of crystals of iminoallantoïn which behaved towards heat in the peculiar way described by Denicke and contained 44.32 per cent of nitrogen; calculated for $C_4H_7N_5O_2 = 44.64$ per cent N.

In other trials it was not equally easy to separate the unaltered compound which may apparently be changed quite readily in the urine. The rabbits were on a fixed diet and responded to the injections with a corresponding increase in the elimination of nitrogen. The purine content of the urine remained unaffected. In every case there was a noticeable increase in the urinary content of oxalic acid, estimated by the method of Autenrieth and Barth.

BEFORE INJECTION. *mgm. per day.*	QUANTITY INJECTED	AFTER INJECTION. *mgm.*
trace	1 gram intravenously	137
trace	1 " "	110
4	1 " intraperitoneally	38
5	0.5 " "	53

Since small quantities of oxalic acid are apparently formed on standing in (alkaline) rabbit's urine containing iminoallantoïn, the data are not conclusive as to its metabolic origin in such experiments.

UROXANIC ACID. By appropriate oxidation of uric acid, it is easy to obtain uroxanic acid



(Behrend: *Ann.d. Chem.*, ccciii, p. 141, 1904.)

in place of allantoïn.[1] Wiener has found that although uric acid will protect rabbits against otherwise toxic doses of benzoic acid, allantoïn and uroxanic acid failed to act in this way.[2] He administered uroxanic acid in doses of 0.5 gram per kilo. We have introduced sodium uroxanate parenterally into rabbits in doses

[1] Cf. Sundwick: *Zeitschr. f. physiol. Chem.*, xli, p. 344, 1904-05.
[2] Wiener: *Arch. f. exper. Pathol. u. Pharmakol.*, xlii, p. 379, 1899.

up to 5 grams without any untoward effects. Attempts to devise a satisfactory method for the recovery of the compound in case it were excreted unchanged, were unsatisfactory. With concentrated sulphuric acid a purple color is developed by uroxanic acid in the presence of indole, skatole, or tryptophane. This is, however, in no way specific. Two definite results may be recorded. The output of purines is not noticeably altered after injecting sodium uroxanate; but the excretion of oxalic acid in the urine was increased in every case. The rabbits were on a fixed diet of carrots. The oxalic acid was estimated by the Autenrieth-Barth method.

UROXANATE INTRODUCED.	DAILY COMPOSITION OF URINE.		
	Volume.	Nitrogen.	Oxalic Acid.
gm.	*cc.*	*gm.*	*mgm.*
None	295	0.608	trace
0.5 = 0.07 gram N, intravenously	240	0.710	6
None	370	0.503	trace
None	225	0.401	2
1.5 = 0.21 gram N, intraperitoneally	323	0.454	14
None	261	0.368	trace
None	208	0.420	3
3.0 = 0.42 gram N, intraperitoneally	332	0.884	49
None	313	0.426	trace

NYLANDER'S REACTION IN THE PRESENCE OF MERCURY OR CHLOROFORM.

By M. E. REHFUSS and P. B. HAWK.

(*From the Laboratories of Physiological Chemistry of the Department of Medicine of the University of Pennsylvania and the University of Illinois.*)

(Received for Publication, January 21, 1910.)

In 1906 Bechhold[1] reported observations which seemed to indicate that urines containing dextrose would fail to yield a positive test for this substance, by Nylander's reaction, in the presence of mercury or chloroform. He found this to be true for diabetic urines as well as for normal urines to which dextrose had been added. Bechhold called attention to the fact that the inhibitory action of mercury would be a fertile source of error in the examination of the urine of syphilitics who were under treatment with mercury as well as in the examination of the urine of other individuals who were in sufficiently frequent contact with the metal to cause its appearance in the urine. He cited, in this connection, the case of a bacteriologist who was accustomed to sterilize his hands with mercuric chloride. The urine of this individual contained sufficient mercury to prevent a positive Nylander reaction even after dextrose had been added. Upon examination Bechhold found this urine to contain 0.056 mgms. of mercury per liter. In the case of syphilitics the inhibition was found to be stronger the greater the number of injections the patient had received. Bechhold further stated that it made very little difference whether the mercuric chloride solution was 1:1000 or 1:10,000 so far as the extent of the inhibition was concerned, provided that uniform volumes were used.

The inhibitory influence of chloroform was also investigated by Bechhold. He claimed that inasmuch as chloroform was frequently used as a urinary preservative this too might cause

[1]Bechhold: *Zeitschr. f. physiol. Chem.*, xlvi, p. 370, 1906.

the drawing of wrong conclusions when urines containing it were submitted to the Nylander reaction.

Immediately upon the appearance of Bechhold's report the authors set about in an attempt to verify his conclusions. This they did not succeed in doing, however, but found practically no inhibitory action when mercury or chloroform was present in the urine. Their findings in this connection were not reported at the time inasmuch as other points in regard to the Nylander reaction were subsequently taken up for study and it was deemed advisable to report upon their entire findings when all phases of the problem were completed.[1] In the meantime Zeidlitz[2] examined into the claims of Bechhold regarding the inhibitory influence of mercury and of chloroform and also failed to confirm the findings of that investigator. He found that the bismuth test gave a positive reaction when applied to urine containing mercury even when the sugar content of such urine was as low as 0.1 per cent.

EXPERIMENTAL.

In all our tests upon the influence of mercury and of chloroform the Nylander reaction was carried out according to the method fully described elsewhere.[3] Briefly it is as follows: To 5 cc. of the solution under examination, in a test-tube, is added one-tenth its volume of Nylander's reagent,[4] and the tube and contents placed in a boiling water-bath. Within a period of five minutes a black end-reaction should be obtained with solutions containing dextrose or other reducing sugars. The authors found it convenient to use a porcelain lined water-bath thus securing an admirable background for observing and comparing the color changes.

In our examination into the claims of Bechhold regarding the inhibitory influence of mercury and of chloroform upon Nylander's reaction we made tests upon three different forms of solution.

[1]Rehfuss and Hawk: *Proceedings of American Society of Biological Chemists*; This *Journal*, May 15, 1909.

[2] Zeidlitz: *Upsala Lakäreforen Forh. N. F.*, xi, 1906.

[3] Rehfuss and Hawk; This *Journal* vii, p, 273, 1910.

[4] *Made by dissolving 2 grams of bismuth subnitrate and 4 grams of* Rochelle salt in 100cc. of 10 per cent potassium hydroxide.

These were, first, the urine of syphilitics who were excreting mercury; second, specimens of normal urine to which mercuric chloride had been added; and third, aqueous solutions of mercuric chloride. It is claimed by Bechhold that there is not the same degree of inhibition with the aqueous solution of mercuric chloride as with the normal urines to which mercuric chloride has been added. We found practically no difference, in neither case being able to detect any appreciable inhibition. Typical observations may be found in Table I, p. 270.

Through the courtesy of Dr. Brooks of the Philadelphia General Hospital we were able to procure specimens of urine from the large venereal wards of that institution. The urine of each syphilitic examined was shown to be free from dextrose, after which the sugar was added and the test applied. All of the cases were receiving mercury in the period during which their urine was collected for examination, yet in no instance was there evidence of the slightest inhibition of the Nylander reaction. Specific data for these tests will be found in Table II, p. 271.

The question of the inhibitory action of choloroform may be dismissed with a few words. The boiling point of chloroform being 61.2° C. it is evident that the technique of our tests (the tube placed for a five minute period in boiling water) must of necessity entirely eliminate the presence of chloroform. We found that the addition of large quantities of chloroform did cause slight inhibition, whereas smaller quantities, e. g., such quantities as would ordinarily be met with in urinary examinations, caused no inhibition. If urines suspected of containing chloroform be boiled for a five minute period previous to testing them by Nylander's reagent, no difficulty will be experienced regarding the inhibitory action of the chloroform. Typical tests on chloroform inhibition are recorded in Table III, p. 272.

CONCLUSIONS.

1. If the Nylander reaction be carried out as suggested in this article the presence of mercury as mercuric chloride does not inhibit the reaction in urinary examination or in the examination of aqueous solutions, even when the mercury is present in fairly high concentration, e. g., 1 cc. of 1:1000 mercuric chloride to 5 cc. of the solution examined.

2. The presence of 0.005 per cent of mercury in the urine, as in the case cited by Bechhold, has, according to our tests, no inhibitory influence.

3. The mercury present in the urine from several syphilitics caused no inhibition of Nylander's reaction. Dextrose added to such urines in small amount was in each instance detected without difficulty.

4. The presence of chloroform in the urine will not prevent the obtaining of a positive Nylander reaction when dextrose is present. In case an excessive amount of chloroform is present it should be removed by subjecting the urine to a preliminary boiling for a period of five minutes before applying the test.

TABLE I.

Influence of Mercury.

SOLUTION EXAMINED.	REACTIONS OBTAINED AFTER BOILING PERIODS OF DIFFERENT LENGTHS, IN MINUTES.			
Normal urine + ½ cc. of 4 per cent dextrose solution was used in each test.	1	2	3	5
1 cc. of 1:1000 mercuric chloride.....	amber	amber	olive	black
0.1 cc. of 1:1000 mercuric chloride....	amber	olive	black	black
0.01 cc. of 1:1000 mercuric chloride..	amber	brown	black	black
Blank test; no mercury.............	amber	brown	black	black

In the following tests water was used instead of normal urine:

1 cc. of 1:1000 mercuric chloride	yellow-brown		brown-black	black
0.1 cc. of 1:1000 mercuric chloride ...	brown-black		black	black
0.01 cc. of 1:1000 mercuric chloride ..	brown-black		black	black
Blank test; no mercury	brown-black		black	black

TABLE II.

Examination of Syphlitic Urine.

DESCRIPTION OF CASE				
No.	Name	White or Colored	Stage of Disease	Condition at time urine was examined
1	T. L.	White	Secondary	Eruption present.
2	J. M. H.	Colored	Secondary	Eruption disappeared under mercury treatment.
3	J. McQ.	White	Tertiary	Gumma in cranial cavity.
4	H. L.	Colored	Secondary	Eruption present.
5	J. McC.	White	Tertiary	Tertiary lesions all over body. Case of precocious syphilis.
6	M. L.	White	Secondary	Eruption beginning to disappear.

TABLE II—*Continued.*

Examination of Syphilitic Urine.

DESCRIPTION OF CASE AND TREATMENT	REACTIONS OBTAINED AFTER BOILING PERIODS OF DIFFERENT LENGTHS (MINUTES.)			
Treatment	1	2	3	5
Ten days, protiodide of mercury gr. $\frac{1}{4}$, t.i.d	amber	brown	black	black
Ten weeks, protiodide of mercury, gr. $\frac{1}{4}$, t.i.d.	amber	dark brown	black	black
Mixed treatment over considerable period........................	amber	dark brown	black	black
Thirteen days, protiodide of mercury, gr. $\frac{1}{4}$, t.i.d.	amber	brown	black	black
Formerly treated in army. Present treatment mercuric chloride gr. $\frac{1}{12}$, t.i.d. and KI gr.X	amber	brown	black	black
Mercury inunctions 2 weeks, and protiodide of mercury 2 weeks...	amber	brown	black	black
Blank test on normal urine	amber	amber	black	black

TABLE III.

Influence of Chloroform.

SOLUTION EXAMINED. Normal urine + ½ cc. of 4 per cent dextrose solution was used in each test.	REACTIONS OBTAINED AFTER BOILING PERIODS OF DIFFERENT LENGTHS. (MINUTES).			
	1	2	3	5
0.1 cc. of chloroform*.............	amber	amber	amber	amber
0.01 cc. of chloroform.............	amber	olive	brown	black
Blank test; no chloroform.........	amber	brown	black	black

In the following tests, after adding the chloroform the urine was boiled for 5 minutes before testing by Nylander's reaction.

0.5 cc. of chloroform..............	amber	amber	olive	black
0.01 cc. of chloroform.............	amber	brown	brown–black	black
Blank test; no chloroform.........	amber	brown	black	black

*Owing to the immiscibility of chloroform and urine as large an amount as 0.1 cc. of chloroform would never occur in a 5 cc. urine sample.

A STUDY OF NYLANDER'S REACTION.

By M. E. REHFUSS and P. B. HAWK.

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(Received for publication, January 21, 1910.)

In connection with our examination into the claims of Bechhold[1] as to the inhibitory influence of mercury and of chloroform upon Nylander's reaction,[2] we made a careful study of this reaction. We investigated the test from five different standpoints as follows: (*a*) Most satisfactory method of performing the test; (*b*) its delicacy; (*c*) the influence of temperature upon the reaction; (*d*) interfering substances: (*e*) its clinical value.

Method of Performing the Test. There has been considerable controversy comparatively recently as to the proper manner in which Nylander's test should be performed. Pflüger[3] claims, as the result of a series of tests in which the urines under examination *were boiled in a water-bath for 15-30 minutes*, that the test is useless inasmuch as more than one-half the normal urines give a positive reaction. Hammarsten[4] defended the test against Pflüger's attack. He performed the test by boiling over a free flame, for 2-5 minutes and claimed that when performed in this way that the test was most satisfactory and served to detect small quantities of reducing material. He calls attention to the fact that Pflüger boiled his tests for a period which is much too long and asserts that the conditions are entirely different when we boil 2-5 minutes over a free flame from those in force when we boil 15 to 30 minutes on a water bath. Hammarsten says it is not hard to understand why normal urines might appear

[1] Bechhold: *Zeitschr. f. physiol. Chem.*, xlvi, p. 370, 1906.
[2] Rehfuss and Hawk: This *Journal*, vii, p. 267, 1910.
[3] Pflüger: *Arch. f. d. ges. Physiol.*, cxvi, pp. 265 and 533, 1907.
[4] Hammarsten: *Ibid.*, cxvi, p. 517, 1907.

to give a positive reaction when subjected to Pflüger's manipulation. Pflüger, in criticising Hammarsten's procedure says that heating over a free flame cannot fail to give gradations in heat and that only that side of the tube in immediate contact with the flame can be perfectly heated. He believes that more uniform heat is secured through his practice of using the water-bath.

In his tests upon the inhibitory action of mercury and of chloroform Bechhold instituted a uniform heating for five minutes upon a boiling water-bath. After making a series of comparative tests of the methods proposed by Pflüger, Hammarsten, and Bechhold we are forced to the conclusion that the five minute boiling period on the water-bath yields most satisfactory results. Therefore we have adopted that procedure in all our work. We have been in the habit of using a large porcelain-lined water-bath, the bottom of which forms an admirable background for observing and comparing the various color changes. The constancy of condition, the fact that several tests may be made simultaneously, together with the very material shortening of time seem to us sufficient reasons for using this modification. In making the test 5 cc. of the urine under examination are placed in a test-tube, 0.5 cc. of Nylander's reagent[1] is added and the tube placed in a boiling water-bath for five minutes. The tube is then removed and examined before a white background. A black end-reaction indicates the presence of reducing sugar in the urine under examination.

The Delicacy of the Test. Inasmuch as there is such great diversity of opinion as to the delicacy of Nylander's test we considered it worth while to investigate this point. Certain textbooks state that the test will detect sugar when present in a concentration of 0.025 per cent whereas other books set values of 0.05 per cent and 0.1 per cent as the delicacy limits for the reaction.

We first tested the delicacy of the reaction when applied to aqueous solutions of dextrose as well as to normal urines to which weighed amounts of dextrose had been added. Many tests of this sort were made, the results from one series of aqueous solutions

[1] We used the following formula for making the reagent: 2 grams bismuth subnitrate; 4 grams Rochelle salt; 100 cc. 10 per cent potassium hydroxide.

being recorded in Table I, p. 281. Inasmuch as a positive reaction is indicated by an actual darkening of the solution following the reduction of bismuth it will be seen that beyond 0.08 per cent the test is no longer available for the detection of dextrose. The yellow end-reaction obtained in solutions more dilute than 0.08 per cent is due to the action of the potassium hydroxide upon the sugar i. e., Moore's reaction. Solutions of lævulose, maltose and lactose were examined in a similar manner. The limit of accuracy in the case of lævulose was found to be 0.07 per cent, whereas the values for maltose and lactose were the same as that determined for dextrose.

The Effect of Temperature upon the Reaction. In these tests 10 cc. of a 1 per cent solution of dextrose were treated with 1 cc. of Nylander's reagent and the tube placed on a water-bath at some constant temperature for a definite period, careful record being made of the period of time necessary to secure the typical end-reaction as well as certain definite intermediate color changes. The data from these tests are given in Table II, p. 282. The color changes are designated as yellow, amber, brown, dark brown and opaque black. It will be noted that the duration of the test varies directly with the intensity of the heat. Thus at a temperature of 74° C. or above, the five minute period of boiling, which we advise, is sufficient to yield the typical black end-reaction in the presence of reducing sugar, whereas a temperature of 67° C. must be maintained for a seven minute period and a temperature of 57° C. requires a period of 23 minutes to produce a like effect.

Interfering Substances. A rather large number of substances have been mentioned by various investigators as interfering with the Nylander test. Among these may be specially mentioned *albumin*, and various *aromatic* and *medicinal substances*, such as rhubarb, senna, antipyrin, salol, kairin, turpentine, etc. When these aromatic and medicinal substances do interfere, and we believe that this contingency is not of frequent occurrence, it is highly probable that they interfere indirectly. It is well known that the administration of many of the substances mentioned will cause an increased output of glycuronates in the urine. If this glycuronate concentration is sufficient a reduction of the bismuth of the Nylander's reagent will occur. Daiber[1] claims that urine

[1] Daiber: *Corresp. Schweizer Aerzte*, xxiv, p. 38.

containing a high indican content will give a positive Nylander test. This point was confirmed by Glaw.[1] Büchner[2] reports a darkening of the reagent when urines rich in uroerythrin or haematoporphyrin are under examination, whereas J. Müller states that every normal urine upon being strongly concentrated through evaporation will yield a positive Nylander reaction. According to Salkowski[3] many very concentrated sugar-free urines such, for example, as those containing chrysophanic acid, yield a dark color when subjected to the Nylander test, while Tyson[4] has frequently observed the formation of a peculiar dirty gray precipitate which he asserts is very confusing.

We have not attempted to verify the claims of the various investigators mentioned. We have, however, studied the influence upon the Nylander reaction, of many normal and pathological urinary constituents. The first tests of this character we made were on albumin. This is universally considered to possess the property of darkening the Nylander reagent even when present in sugar-free urines. The color formed is a deep amber, cherry or brown and in the presence of the correct phosphate concentration a characteristic reddish-brown color results. An actual *black* color never results, however, when urines containing albumin are examined and such color as is formed is believed to be due to the splitting off of unoxidized sulphur from the protein and the subsequent formation of bismuth sulphide. In our albumin tests we first studied the influence of aqueous albumin solutions of varying strengths upon Nylander's reagent. Typical results are shown in Table III, p. 283. An examination of the data here tabulated will indicate that it is necessary to have a fairly high albumin concentration before a color at all similar to that secured in the presence of sugar is obtained.

Our experiments on testing for sugar in the presence of albumin demonstrate a very interesting fact. This is to the effect that albumin when present even in moderate amount in urines containing sugar renders the detection of this sugar by the Nylander test exceedingly difficult if not totally impossible through the

[1] Glaw: *Deutsche med. Zeit.*, xvi, p. 689.
[2] Büchner: *Münch. med. Woch.*, xli, p. 991
[3] Salkowski: *Practicum*: p. 181, 1900.
[4] Tyson: *Practical Examination of Urine*, Tenth Edition, p. 91.

formation of a peculiar colloidal solution. The data given in Table IV, p. 284 will demonstrate this. This same phenomenon was noted repeatedly in similar tests. It will be noted after 1 or 2 minutes boiling that the tubes containing albumin possess a darker tint than do those tubes to which no albumin was added. However subsequent boiling has no material effect upon this color whereas in the case of the urines containing sugar the color progressively deepens until the typical opaque black end-reaction is reached. Upon standing 24 hours the tubes containing albumin exhibit no change in color and the formation of no precipitate while the sugar tubes show an opaque black precipitate with a pale yellow supernatant liquid. Evidently in the presence of the albumin some colloidal reaction has taken place such as to prevent the further progress of the true Nylander reaction although sufficient sugar is present to yield the typical end-reaction in the absence of albumin. This phenomenon may be due to the fact, suggested to us by Professor John Marshall, that a part of the albumin which has not undergone cleavage holds the bismuth sulphide in colloidal solution. Many experiments showed results similar to those indicated above. Reference to Bechhold's[1] last table will show a similar condition with the exception that he used small amounts of serum. We were unable to verify this and obtained the colloidal solution only when large amounts of albumin and small amounts of sugar were used. Larger amounts of sugar eventually overcame the tendency toward the formation of the colloidal solution.

Peptone in the presence of Nylander's reagent yields a color similar to that obtained in the case of albumin. (Table III, p. 283). However ,when we come to test for sugar in the presence of these proteins we find a difference.. Whereas in the case of albumin, as above cited, colloidal solutions resulted instead of the typical end-reaction no such phenomenon was observed in the case of peptone. (Table IV, p. 284). From what we know of colloidal solutions this is right in line with what we would expect. Peptone being dialyzable cannot of course hold the bismuth in a colloidal state and it therefore precipitates just as it does in the absence of protein material.

[1] Bechhold: *Loc. cit.*

Further pathological urinary constituents, other than albumin and peptone whose influence upon Nylander's reaction we studied, included *tyrosin*, *leucin*, *cholesterol*, and *cystin*. None of these constituents yielded a reaction simulating the sugar reaction in the absence of sugar and in the presence of sugar none of them except tyrosin and leucin exerted any inhibitory influence. The inhibition produced by tyrosin and leucin was very slight. The reaction was delayed a trifle but the typical end-reaction was ultimately obtained. According to Hammarsten homogentisic acid and alkapton do not yield any reaction resembling the sugar reaction although the alkapton does cause the formation of a flocculent brownish precipitate. We could not obtain any urine containing these substances and hence did not study their action.

The influence of excessive amounts of several normal urinary constituents was studied. Among these were *urea*, *uric acid*, *sodium urate*, *potassium urate*, *creatinin* and *phosphates*. There was no reduction in any case. This fact emphasizes the advisability of using Nylander's reaction in testing for sugar in urines which contain a high concentration of uric acid or creatinin inasmuch as it is well known that these substances when present in the correct amount may cause the drawing of wrong conclusions when such urines are examined by the copper tests.

Our next tests were made on several acids the greater number of which are regularly used internally or externally either as such or in the form of some derivative. In each instance care was taken to see that the alkalinity of the test was not lowered. Table V, p. 285, gives the results of tests of this character. The acids tested included *acetic*, *trichloracetic*, *tannic*, *boric*, *tartaric*, *picric*, *carbolic*, *arsenious*, *benzoic*, *salicylic*, *chromic*, and *pryogallic*. After demonstrating that these acids would not of themselves cause a similar end-reaction to that produced by sugar a series of tests were made with the idea of detecting any inhibitory influence they might exert when present in urine containing sugar. Data from these tests are given in Table V, p. 285. An examination of this table will show that of all the acids studied only three, i. e., trichloracetic, chromic and pyrogallic exerted any inhibitory action upon the Nylander test. The reaction with pyrogallic acid is the same (red-brown) no matter whether the urine tested be sugar-free or contain a high concentration of the carbohydrate.

The urine from a case in which considerable calcium sulphide had been administered was examined but failed to yield a positive Nylander reaction. The influence of urotropin is also of considerable interest. This drug yields small quantities of formaldehyde in the urinary tract and according to our experience this aldehyde reduces Nylander's reagent. The claim has been made by Abt[1] that the urine of patients receiving comparatively large quantities of urotropin gives a pronounced reduction of Fehling's solution and has but slight action on the alkaline bismuth tests. However, Weitbrecht[2] has recently reported that the sugar-free urine of a patient to whom urotropin was administered was found to reduce Nylander's reagent. This was attributed to formaldehyde resulting from partial decomposition of hexamethylenetetramine in the organism.

Clinical Value of Nylander's Reaction. Pflüger,[3] as before stated, claims to have secured a positive Nylander reaction when testing urines known to be sugar-free. He therefore prefers the copper tests over those of bismuth. Kistermann[4] on the other hand claims that those urines which reduce the Nylander reagent also reduce the copper tests. As pointed out on p. 273 the communication of Hammarsten seems to furnish ample demonstration of the inaccuracy of Pflüger's methods. The latter exposes the test to the heat of the water-bath for a period of from fifteen to thirty minutes. Such procedure cannot fail to demonstrate at least doubtful if not actually positive reactions with many of what are claimed to be normal urines.

In Table VI, p. 286, we have tabulated some data secured from prolonged boiling of normal urines on a water-bath in the presence of Nylander's reagent, according to Pflüger's methods. Of the urines mentioned, Nos. 652-656 represent perfectly normal urines, and yet the majority of them yielded a positive Nylander test after sufficient boiling. The point to be noted however is that at the end of a five minute boiling period, the period prescribed by us, none of them showed signs of any reducing action. Numbers 657 and 658 represent morning and evening urine sam-

[1] Abt: *Archives of Pediatrics*, xxiv, p. 275, 1907.
[2] Weitbrecht: *Schweiz. Wochschr.*, xlvii, p. 577, 1909.
[3] Pflüger: *Loc. cit.*
[4] Kistermann: *Deutsch. Arch. f. klin. Med.*, l, p. 423, 1892.

ples of a person whose urine at this stage was supposed to be perfectly normal and who was subjected to an iridectomy preliminary to the cataract operation. Several weeks after the cataract operation he developed glycosuria which later became fairly well marked. It will be noted on reference to the table that Fehling's test is marked "doubtful" in the case of No. 658 whereas Nylander's test yielded a positive reduction at the end of a half-hour boiling period. It is, of course, impossible to determine in this instance whether the reduction was due to the presence of a trace of sugar or was due to the same tendency which causes reduction in normal urines. Number 659 represents a case which developed nephritis about one year later.

Our results with Nylander's reagent, in comparison with other well known sugar tests, confirm us in our opinion that it is one of clinical value. In the examination of over seven hundred urines there were only two instances in which Nylander's reaction was positive and Fehling's test negative. In the first instance there was a very marked reduction which was assignable to no apparent cause, whereas in the second instance there was a very slight positive reaction. We believe with Hammarsten and Kistermann that a negative Nylander reaction affords evidence that the urine, from a clinical standpoint, may be considered sugar-free. The statement of the latter to the effect that any protein-free urine of acid reaction which gives a negative Nylander's test may safely be said to be *sugar-free* in a clinical sense, seems to be justified.

TABLE I

Delicacy of Nylander's Reagent for Dextrose.

PERCENTAGE OF DEXTROSE	LENGTH OF BOILING PERIOD (MINUTES)					POSITIVE OR NEGATIVE (+ or —)
	½	1	1½	2	5	
5	amber	amber	deep amber	deep amber	opaque black	+
2.5	yellow	amber	deep amber	deep amber	opaque black	+
1.0	pale yellow	amber	deep amber	yellow brown	opaque black	+
0.5	pale yellow	amber	deep amber	yellow brown	opaque black	+
0.25	very pale yellow	yellow	amber	deep amber	opaque black	+
0.10	colorless	pale yellow	yellow	amber	brown black	+
0.09	colorless	very pale yellow	pale yellow	yellow	brown black	+
0.08	colorless	very pale yellow	pale yellow	yellow	brown black	+
0.07	colorless	very pale yellow	pale yellow	yellow	deep amber	—
0.06	colorless	very pale yellow	pale yellow	yellow	yellow	—
0.05	colorless	almost colorless	pale yellow	pale yellow	yellow	—
0.04	colorless	colorless	pale yellow	pale yellow	yellow	—
0.03	colorless	colorless	very pale yellow	very pale yellow	very pale yellow	—
0.02	colorless	colorless	colorless	almost colorless	almost colorless	—
0.01	colorless	colorless	colorless	colorless	colorless	—

TABLE II

Influence of Temperature. (Centigrade)

MINUTES HEATED	57°	58°	59°	60°	62°	64°	67°	68°	70°	72°	74°
1⅓							yellow				
1¾				yellow							
2⅓	yellow							amber			brown
3										brown	
3¾						amber					dark brown
4½					amber					dark brown	
5				amber					brown		opaque black
5¼			amber							opaque black	
5½								brown	dark brown		
5¾								dark brown			
6		amber					brown		opaque black		
6¼							dark brown				
6¾								opaque black			
7	amber					brown	opaque black				
7¾					brown	dark brown					
8⅓					dark brown	opaque black					
9											
9¾					opaque black						
10½				brown							
11½				dark brown							
12½			brown	opaque black							
12¾			dark brown								
15			opaque black								
19		brown									
20⅓	brown	dark brown									
21	dark brown										
21⅓		opaque black									
23	opaque black										

TABLE III

Influence of Albumin and of Peptone upon Nylander's Reaction

(*a*) ALBUMIN.

VOLUME OF 2 PER CENT ALBUMIN SOLUTION IN 5 C. C.	BOILING PERIOD (MINUTES)			POSITIVE OR NEGATIVE (+ OR —)
	1	2	5	
2 cc.............	amber	amber	amber brown	—
1 cc.............	yellow	yellow	amber brown	—
0.5 cc.............	yellow	yellow	yellow	—
0.1 cc.............	colorless	colorless	colorless	—
0.01 cc.............	colorless	colorless	colorless	—

(*b*) PEPTONE.

VOLUME OF 2 PER CENT PEPTONE SOLUTION IN 5 C. C.	BOILING PERIOD (MINUTES)			POSITIVE OR NEGATIVE (+ OR —)
	1	2	5	
2 cc.............	yellow	amber	deep amber	—
1 cc.............	pale yellow	pale yellow	pale yellow	—
0.5 cc.............	very pale yellow	very pale yellow	very pale yellow	—
0.1 cc.............	colorless	colorless	colorless	—
0.01 cc.............	colorless	colorless	colorless	—

TABLE IV

Influence of Albumin and of Peptone in presence of Sugar.

(*a*) ALBUMIN

CONSTITUENTS	BOILING PERIOD (MINUTES) 1	2	5	POSITIVE OR NEGATIVE (+ OR −)
5 cc. 2 per cent albumin solution + 0.5 cc. 2 per cent dextrose solution..	deep amber	deep cherry	deep cherry (colloidal)	−
5 cc. 2 per cent albumin solution + 0.5 cc. 2 per cent dextrose solution..	deep amber	deep cherry	deep cherry (colloidal)	−
Check test. 5 cc. water + 0.5 cc. 2 per cent dextrose solution	pale yellow	amber	opaque black	+
Check test. 5 cc. water + 0.5 cc. 2 per cent dextrose solution	pale yellow	amber	opaque black	+

(*b*) PEPTONE.

CONSTITUENTS	1	2	5	POSITIVE OR NEGATIVE (+ OR −)
5 cc. 2 per cent peptone solution + 0.5 cc. 2 per cent dextrose solution..	amber	amber	black	+
2 cc. 2 per cent peptone solution + 0.5 cc. 2 per cent dextrose solution..	amber	deep amber	black	+
1 cc. 2 per cent peptone solutition + 0.5 cc. dextrose solution..........	yellow	brown	black	+
0.5 cc. 2 per cent peptone solution + 0.5 cc. dextrose solution..........	yellow	brown	black	+
0.1 cc. 2 per cent peptone solutio + 0.5 cc. 2 per cent dextrose solution..	yellow	dark brown	black	+

TABLE V

Influence of Acids.

Acid	Influence on Nylander's Reagent in Presence of Sugar. Boiling Period (Minutes.) 1	2	3	4	5
Trichloracetic	yellow	amber	amber	amber	amber
Acetic	yellow	amber	brown	black	black
Picric	cherry-red	mahog.-red	mahog.-red	mahog.-black	mahog.-black
Tannic	yellow	amber	brown	brown black	black
Boric	pale yellow	yellow	brown	brown	brown black
Tartaric	pale yellow	yellow	brown	brown black	black
Carbolic	yellow	amber	brown	brown black	black
Chromic	green	olive green	translucent olive green	translucent olive green	translucent olive green
Benzoic	yellow	dark amber	brown	brown black	black
Arsenious	yellow	brown	brown black	black	black
Salicylic	yellow	brown	brown black	black	black
Pyrogallic	amber	orange	red brown	red brown	red brown

TABLE VI.

Influence of Prolonged Boiling of Normal Urines.

All urines were acid in reaction and none contained albumin or sugar.

No.	Fehling's Test	Boiling Period (Minutes).						Positive or Negative. (+ or −)
		5	10	15	30	45	60	
652	negative	yellow	yellow	yellow	gray-brown, ppt.	gray-brown, ppt. increased	brown-black	+
653	negative	yellow	deep amber	deep amber	deep amber, gray ppt.	deep amber, gray ppt.	deep amber gray ppt.	−
654	negative	yellow	brownish ppt.	brown-black	deep brown-black	deep brown-black	opaque black	+
655	negative	amber	amber	amber,	amber	amber	amber	−
656	negative	yellow	yellow, gray ppt.	yellow, gray ppt.	gray-black	brown black	black	+
657	negative	amber	amber	amber	amber	amber	amber	−
658	doubtful	amber	amber	brown-black	black	black	black	+
659	negative	amber	amber	brown-black	brown-black	brown-black	black	+

EFFECTS OF SOLUBLE SALTS ON INSOLUBLE PHOSPHATES.[1]

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INTRODUCTION.

This study has been taken up from two viewpoints. First, a study of field and pot experiments, with a view of determining, if possible, the indirect action of various salts when used as fertilizers; to ascertain if the increase in yield obtained from the use of a fertilizer is not due in part to the solvent action which it exerts on the comparatively insoluble phosphorus in the soil. Second, to study in the laboratory, the solvent action of the more common salts on phosphates, using distilled water as a standard.

That some fertilizers have an indirect effect has long been an acknowledged fact. It is no longer believed, that the increase in yield obtained from the use of one ton of barnyard manure, is alone due to the addition of the two or three pounds of phosphorus and the ten or twelve pounds each of nitrogen and potassium which it contains; but no small part of the increase is due to the liberation of more plant food. This (1) is brought about by the decaying of organic matter with the formation of various acids which in turn act as solvents on insoluble plant food already in the soil. The benefits obtained from the use of gypsum and lime are due not only to the neutralizing of acid and supplying of calcium, but it is acknowledged that they act indirectly by liberating potassium. From certain results obtained in various experiments, which will be considered later, it seems as if this may be due in part, to the liberation of phosphorus from its less soluble forms.

[1] This investigation was suggested by Dr. C. G. Hopkins of the University of Illinois, and many valuable suggestions were received from him.

The theory of the liberation of phosphorus by the addition of various soluble salts to the soil is not a new one for some writers speak of it as an established fact. This was understood in a general way by Justus von Liebig, for in his article (2), "Some Points in Agricultural Chemistry," he says: "These salts (speaking of the ammonium salts) contain an acid which exerts an action on the constituents of the soil, an action which is not exerted by pure ammonia. The acids of the ammoniacal salts render the earthy phosphates more soluble in water than they would otherwise be."

A. Stood (3), in a study of the effects of salt water on the soil, attributes part of the bad effects of salt water on land to the rendering soluble the phosphates which are subsequently washed beyond the plant roots.

For some time in England the agricultural investigators were divided into two schools. One of these claimed that a great increase in yield could be obtained by the use of an insoluble phosphate; while the other claimed that very little, if any increase in yield could be obtained by its use. Each supported his claim by actual field tests. This difference in results was explained by E. Waldt (4) as being due to the salt which in some cases had been used in connection with the phosphate. Some salts, he claims, when used in connection with a phosphate tend to render it more soluble. Especially does he attribute this property to the various nitrates.

Coming down to the present time we have the statements of Hilgard (5) that lower percentages of potassium,[1] phosphorus and nitrogen are adequate, when a large proportion of lime carbonate is present. He states further, that a high percentage of lime carbonate may offset a small percentage of phosphorus apparently by bringing about greater availability. Again, in summing up the chemical actions of carbonates he states that carbonates liberate phosphorus and potassium from insoluble forms.

Wagner (6) found that some of the benefits which result from the use of sodium nitrate are due to its rendering more soluble certain phosphates.

[1] Throughout this article potassium and phosphorus have been stated as the elements in place of potash and phosphoric acid. When quoting from others this change has been made.

SALTS CONTAINING NITROGEN COMPARED WITH DRIED BLOOD AS A SOURCE OF NITROGEN.

As a source of nitrogen, the nitrate of soda is usually considered to produce a much better yield than an equivalent amount in the form of dried blood. This is well illustrated in a series of pot experiments by Voorhees (7). The experiments were carried out in the year 1901 and 1902. Sodium nitrate and dried blood were the sources of nitrogen; equivalent amounts of nitrogen being used in each case. As an average of two years and of three experiments each year, dried blood gave a yield of 106.8 grams while sodium nitrate yielded 110.4 grams of dry plant.

Patterson (8) obtained similar results with field tests as the following table will show. Each result is the average of two years' work.

CROP.	FERTILIZER.	GRAIN.	FODDER
		bus.	*lb.*
Corn	Sodium nitrate	61.5	3050
Corn	Ammonium sulphate	52.7	2625
Corn	Dried blood	49.7	2812
Wheat	Sodium nitrate	14.9	2030
Wheat	Ammonium sulphate	14.3	1830
Wheat	Dried blood	9.0	884
Hay	Sodium nitrate		4150
Hay	Ammonium sulphate		1900
Hay	Dried blood		2550

The above shows that corn with sodium nitrate yielded 11.8 bushels and with ammonium sulphate 3 bushels more than it did with an equivalent amount of nitrogen in the form of dried blood. Wheat with sodium nitrate yielded 5.9 bushels and with ammonium sulphate 5.3 bushels more than with the dried blood. Hay produced 1600 pounds more with sodium nitrate and 650 pounds less with the ammonium sulphate than with the dried blood.

A. Muntz (9) also shows that sodium nitrate produced better yields than an equivalent amount of nitrogen in the form of dried blood.

Therefore under ordinary conditions sodium nitrate is more effective than ammonium sulphate, which, however, is more effective than an equivalent amount of dried blood. This, however, in the case of sodium nitrate and ammonium sulphate is reversed under certain conditions as will be shown in the following discussion.

THE RELATIVE VALUE OF SODIUM NITRATE AND AMMONIUM SULPHATE WHEN USED IN CONNECTION WITH AN INSOLUBLE PHOSPHATE.

It seems likely that this difference in value of the two fertilizers may be due in part to some indirect effect of the salt. This is at least indicated by the difference in action of ammonium salts and sodium nitrate when used in connection with insoluble phosphate. On ordinary soil sodium nitrate is usually conceded to give the better yield (10). However, when an insoluble phosphate is used in connection with the nitrogenous manure the ammonium salt gives a larger yield than the sodium nitrate or nitrogen from organic sources. Besides, plants grown with the ammonium salts contain a larger percentage of phosphorus.

A great number of experiments have been carried out to ascertain the relative value of insoluble phosphates. A few of them are as follows: Jameson (11) conducted a series of experiments with turnips, in which a soluble and insoluble phosphate was used. In one series a soluble salt was used with the phosphates, in the other phosphates were used alone. As an average of forty experiments he obtained with the soluble phosphate 15,133 kilos per acre, while with the insoluble phosphate he obtained but 14,663 kilos. per acre. This is a difference of 470 kilos in favor of the soluble phosphate. When the same phosphates were used in connection with ammonium sulphate in a series of twelve experiments, the yield was as great with the insoluble as with the soluble phosphate. Now, when the same two phosphates were used in connection with sodium nitrate the soluble gave a yield of 22,240 kilos, while the insoluble gave but 20,525 kilos. It may be seen that there was 1715 kilos per acre in favor of the soluble phosphate. When the above facts are taken into consideration remembering that nitrates are usually the best form of nitrogen it would seem that

the ammonium sulphate had some effect on the insoluble phosphate.

Krocker and Grahl (13) obtained as large a yield with insoluble phosphate as with the soluble phosphates when ammonium sulphate was used in connection with the phosphate.

A number of experimenters have noted the above facts and carried out various experiments to ascertain the nature of this effect. One of the prominent workers in this field is H. G. Soderbaum (14) from whose work the following table was taken. The crop grown was oats.

FERTILIZER.	YIELD OF GRAIN.
	grams.
Soluble phosphate	16.1
" " + sodium nitrate	61.9
Bone meal + sodium nitrate	49.4
" " + ammonium nitrate	57.9
" " + ammonium sulphate + sodium nitrate	55.9
" " " "	62.9
" " + urea	53.1
" " " + albumin	51.1

The yield with the bone meal and ammonium sulphate was as great as that with the soluble phosphate and sodium nitrate. While not so good as ammonium sulphate, ammonium nitrate gives a better yield than sodium nitrate or the organic manures. It may be seen that when used with slightly soluble phosphate sodium nitrate is no better than the organic manure.

This greater yield with the ammonium salts has been attributed to a physiological action of the ammonium sulphate on the plant, and not to a solvent action on the insoluble plant food. The experiment of Schulov (15), however, seems to indicate that it is due to the latter cause. In these experiments there were two sets of pots, one set in which the ammonium salts and the phosphate were thoroughly mixed; while in the other set the ammonium salts and phosphate were separate but both were accessible to the plant. Where the fertilizers were mixed there was a much larger yield obtained with the ammonium nitrate than with the sodium nitrate. However, where the nitrogen and phosphorus were separate the yield with each fertilizer was the same.

The work of Brooks (16), at the Hatch Experiment Station on the comparative value of potassium chloride and potassium sul-

phate, is of interest in this connection. In a three years test on potatoes, there was an average of 22.1 bushels more where the sulphate was used than where the chloride was used.

We must bear in mind that this beneficial effect of the sulphate may be due in part to the plant food which it supplies in the form of sulphur. That plants require the presence of sulphur to make a healthy growth is well known. Bogdanov (17), Haseloff and Goosel, König and others have found that part of the beneficial effects obtained from the use of a sulphate is due to the sulphur which acts as a plant food. With Brook's experiment, however, there was an increase in the starch in the potato, and Seisl and Gross (59) found that leaves of potatoes which were rich in starch invariably contained more potassium and phosphorus than ones low in this constituent.

That the increase is not due entirely to the action of the sulphur as a plant food is further shown when we make a study of the phosphorus in the plants which have been grown with various fertilizers. A Swedish (18) investigator found that oats grown with an ammonium salt contained .397 per cent of phosphorus while those grown without the ammonium salt contained .375 per cent phosphorus. D. Praimschukow (22) found that buckwheat grown with insoluble phosphate and sodium nitrate contained .105 per cent of phosphorus; while that grown with the same phosphate, in connection with ammonium nitrate contained .253 per cent phosphorus. Barley grown with ammonium nitrate contained .10 per cent more phosphorus than that grown with sodium nitrate.

The experiment which illustrates this best is the mixed herbage of permant grass land by Lawes and Gilbert (23). As an average of eighteen years, the plot which received no manure had 2.288 per cent phosphorus in the ash of the grass, while that which received ammonium salts had 2.790 per cent. This is .502 per cent more phosphorus in the ash of the plants which had received ammonium salts. Besides, this increase there was a greater yield of hay on the manured than on the unmanured so that the total phosphorus taken up would be considerably more in the one case than in the other.

Again, take the plot in the Rothamsted (24), experiment with wheat, which received ammonium salts and compare it with tha

unmanured plot. The plot which received no manure produced as an average of 42 years $12\frac{3}{4}$ bushels of wheat per acre, while the one receiving ammonium salts averaged $19\frac{1}{2}$ bushels per acre. We find that 11.64 pounds of phosphorus were taken up by the crop on the unmanured land; while the one receiving ammonium salts gave as an average 14.5 pounds per acre. From this we see that there was 2.93 pounds per acre more taken up where the ammonium salts had been used. However, in this case the percentage composition is no higher in the manure gram.

That the phosphorus is more soluble on the plots which have been manured with ammonium salts is further shown by a comparison of the drainage water (25) of the two plots. The unmanured plot had as an average .275 parts per million of phosphorus in the drainage water. The plot which received ammonium salts had as an average .629 parts per million of phosphorus, thus showing that the drainage water from the manured plot is richer in phosphorus than from the unmanured.

The above facts show that of the nitrogen compounds ammonium nitrate is the most effective in causing the assimilation of phosphorus from insoluble phosphates. The ammonium sulphate stands next, while sodium nitrate has little if any effect. This is well illustrated by the work or D. Priamschukow (12) who made some very thorough tests in which he used phosphorite with various nitrogenous salts. The following table gives the results he obtained with oats.

SOURCE OF PHOSPHORUS:	PHOSPHORITE.						KH_2PO_4
SOURCE OF NITROGEN.	$NaNO_3$	$3/4NaNO_3$ $1/4(NH_4)_2SO_4$	$1/2NaNO_3$ $1/2(NH_4)_2SO_4$	$1/4NaNO_3$ $3/4(NH_4)_2SO_4$	NH_4SO_4	NH_4NO_3	$NaNO_3$
Yield in grams..	6.9	22.0	20.5	19.2	1.6	18.9	19.8
Per cent phosphorus.........	.039	.131	.249	.402	.637	.244	.231
Total phosphorus in plant mg.	2.707	30.815	50.99	77.00	9.21	46.02	45.75

The yield was as great with the ammonium nitrate and phosphorite as it was with the soluble phosphate and sodium nitrate.

The percentage of phosphorus in the two crops was also the same. Similar tests were made using barley, buckwheat, peas, flax, vitch and in every case where the ammonium nitrate was used with the phosphate the yield was practically as large as it was with the soluble phosphate. The percentage of phosphorus in the plant was also high with ammonium salts. Thinking this may be due to nitrification he carried on tests in sterile cultures where nitrification did not occur and found that even then the ammonium nitrate increased the assimilability of phosphorus of insoluble phosphates.

SODIUM CHLORIDE AS A FERTILIZER.

Sodium chloride, when used as a fertilizer, seems to vary under different conditions. Some experimenters obtain a good yield from its use, others obtain just as good a yield without it. It seems as if there must be some cause for this difference and it may be due to its indirect effects on other plant food through a physiological action on the plants.

F. Stoup (26), in an article on sodium chloride as a manure, attributes the benefit derived from its use as due to its decomposing insoluble plant food. If this be the correct theory we can account for yields such as those obtained by Dr. Voeckler (27). As an average of five experiments, on land which had been manured with common salts, the yield of mangels was 36,060 pounds. On the adjoining unmanured ground there was but 26,035 pounds; a difference of a little over 10,000 pounds due to the use of common salt. Now if the land was rich in insoluble plant food and the chloride was able to liberate it we could expect a large yield. On the other hand, if the land had been poor in unavailable plant food no good result would have followed its use. Wheeler (18) seems to have established the fact that sodium chloride cannot to any great extent take the place of potassium salts. However, he does think that sodium chloride can liberate phosphorus from insoluble forms as the following will show: "It may, however, be stated here that sodium salts seem to liberate phosphorus and potassium so that under certain circumstances they may act as an indirect manure." In a later report (29) he shows that the percentage of phosphorus

in a plant is increased by the use of a sodium salt. With radish this was, in some cases, as much as .052 per cent more in the crop from land which had received a full ration of sodium over that which received but a part ration. In the case of turnips there was a difference of .121 per cent; the beets .035 per cent; the carrots .074 per cent; while in the case of the chickory the results are practically the same in the crop from the manured and unmanured land. The report contains many more cases in which the sodium salt increased the phosphorus in the plant. However, the laboratory tests which have been made on phosphates show that sodium chloride depresses the solubility of a phosphate. If we understood the metabolism of a plant, and plants were grown on the soil containing a phosphate and the chloride, we may find that sodium chloride indirectly rendered the phosphate more assimilable as the above facts tend to show.

EFFECTS OF LIME ON PHOSPHORUS.

It seems to be a well established fact that lime will under certain conditions liberate phosphorus from the soil. The more recent work on this subject is that of the Rhode Island Experiment Station. B. L. Hartwell and J. W. Kellogg (30) in speaking of their turnip experiments, with and without lime, say: "The crop of turnip roots from the limed plot which had received finely ground bone was 62 per cent greater than from the corresponding unlimed plot and the per cent of phosphorus in the dry matter of the roots was 0.378 from the limed plot and 0.351 from the umlimed one. Again, the increase in the crop of turnip roots from the limed plot to which slag meal had been added was 34 per cent as compared with the unlimed plot and phosphorus in the dry matter of the roots was 0.324 per cent from the lime plot and 0.309 per cent from the unlimed one. These increases in the percentage of phosphorus in the turnip roots grown upon the limed plots furnish some evidence that more of the phosphorus in the plots was assimilable."

These same authors made a test of the phosphorus in the soil by extracting with and without lime. They found more phosphorus in the solution from the soil which had been treated with lime than that which had not.

In this same line are the experiments of O. Kellner (31) and his co-workers. They found in the field and laboratory test that phosphorus was liberated by the use of lime.

Again, the work of Sutherst (32) shows that insoluble phosphates of the soil become much more soluble when treated with lime. Especially was this true in the case of the ferric phosphate. The solvent action was not found to take place when calcium carbonate was used.

The work of H. J. Wheller and G. E. Adams, in "A Test of Nine Phosphates with Different plants," is full of illustrations in which lime has been effectual in the liberation of phosphorus. They even claim that it may be of value when a soluble phosphate is used as may be seen from the following: "The results seem to indicate that in a soil deficient or devoid of carbonate of lime and well supplied with the oxides of iron and aluminum, liming may extend the period of efficiency of the soluble phosphates possibly by combining with such of the phosphorus at once, and thus holding it in more assimilable combinations than if it were possible for it all to unite immediately with the iron and aluminum oxides."

EFFECT OF IRON SULPHATE ON PHOSPHORUS.

Some writers have made great claims for iron sulphate as a fertilizer. A goodly number of these claims have been made by persons who would profit by its sale. Even when we ignore these cases, there are still cases in which it has produced good results.

The man who made the greatest claim for this, and backed his claim with actual field tests, was Griffiths. (33) He made tests with it as a manure on a number of crops. The yields which he obtained were much greater with than without it. Especially was this true with beans, turnips, mangels, potatoes, meadow hay, and grass. With wheat and other grains the yield did not appear to increase with the application of the iron sulphate. Griffiths attributed this increase in yield to the supplying of iron to the plant. For he found considerably more iron in the plants, which had been grown on land manured with the iron sulphate than those grown on the unmanured land. The increase may be due in part to this cause, but a study of the phosphorus of the crop would seem to indicate that there is also another cause.

As an average of three years the bean plants grown on land manured with iron sulphate contained 17.95 per cent (33) of phosphorus in the ash; while those grown on adjoining unmanured land contained but 16.47 per cent of phosphorus. In the ash of the pods alone, there was 15.78 per cent phosphoric acid in those from the manured land and 15.03 from the unmanured. The phosphorus in the seed from the manured and unmanured land was the same. With turnip leaves it stood 3.03 per cent in the ash of those grown with the manure and 1.84 per cent in those grown without it. In the roots there was 0.61 per cent more phosphorus in the ash of those grown with sulphate than in those grown without it. Meadow hay had 3.39 per cent phosphorus in the ash of that grown on land manured with the sulphate and 2.34 per cent in that grown without it. Practically the same relationship exists in grass grown under the two conditions. Mangels, 1.00 per cent, potatoes, 1.01 per cent, beet roots, 1.18 per cent more in the ash of those grown on land manured with sulphate than those grown of land not thus manured. Wheat was about the same on manured and unmanured land.

Boetet and Paturel (35) obtained an increase in the crop due to the use of iron sulphate but differ from Griffiths in not finding a greater amount of iron in the plants grown on land manured with iron sulphate.

The Hill (36) experiments in England are of the same type. Wheat was grown in pot experiments with and without iron sulphate. The pot which received no sulphate yielded 35.38 grams of wheat and straw, while an average of the three manured pots was 36.48 grams. The yield was greatest on the pot which received at the rate of 100 pounds of iron sulphate per acre.

W. P. Brooks (55) obtained a larger yield of soy beans on land manured with iron sulphate than on unmanured land. However, he did not find a deeper green in the plants on the manured land as did Griffiths.

Taking into account the above facts it would seem as if under some conditions iron sulphate can assist in the assimilation of phosphorus. Just how this is brought about is hard to say for extraction tests show that iron sulphate renders phosphates less soluble.

CALCIUM SULPHATE.

Calcium sulphate is the most powerful land stimulant we have. This is mostly caused by its liberation of plant food, especially potassium. However, there are some experiments which tend to show that it affects the phosphorus of the soil.

The experiments carried on at Tokyo (37) show that rice yielded better and had a better color when grown on land manured with gypsum.

The analysis made by Baussingault and quoted by Storres (38) shows a greater amount of phosphorus in clover taken from land manured with gypsum. The phosphorus in the clover from the manured land was 10.57 kilos; that from the unmanured 4.80 kilos. The following year although no more manure was applied the phosphorus from the hay grown on the manured land was 6.93 kilos more than from the unmanured.

Pfeffer (56) states that Knop found that when seeds are in water containing calcium sulphate, the calcium of the salt is absorbed in a somewhat greater amount than the acid. If this be true it is easy to see how calcium sulphate can assist in the assimilation of phosphorus, even though the phosphates are found to be less soluble in a calcium sulphate solution.

EFFECT OF OTHER SULPHATES ON PHOSPHORUS.

The sulphates seem to act very strongly on the insoluble phosphorus of the soil. Where there is a lack of available phosphorus, the sulphates produce yields over and above the chlorides or nitrates with the exception of ammonium nitrate. The Rothamsted experiments illustrate this in a very striking manner. The following table gives the yield of wheat from plots 3, 11, 12, 13 and 14.

Table showing the average yearly yield of wheat for 51 years on the Rothamsted Experimental Farm.

PLOT NO.	TREATMENT.	AVERAGE OF 48 YEARS 1852-92.	1899.	1900.	1905.
3	Unmanured continuously	$12\frac{7}{8}$	12	$12\frac{1}{4}$	18.0
11	400 lb. amm. salts 350 lb. superphosphates	$24\frac{1}{4}$	$21\frac{1}{4}$	$18\frac{1}{4}$	18.9
12	400 lb. ammonium salts, 350 lb. superphosphates, $366\frac{1}{2}$ lb. Na_2SO_4	30	$28\frac{3}{4}$	$24\frac{3}{4}$	30.5
13	400 lb. amm.-salts, 350 lb. superphosphates 200 lb. K_2SO_4	$31\frac{1}{2}$	$26\frac{3}{4}$	$28\frac{1}{8}$	39.4
14	400 lb. amm.-salts 350 lb. superphosphates 280 lb. Mg. SO_4	$30\frac{3}{8}$	$28\frac{1}{4}$	$23\frac{1}{4}$	26.0

It may be seen from the above table that the plot which received sodium sulphate gave as an average 30 bushels per acre, or $5\frac{1}{2}$ bushels more than plot 11 which with the exception of the sodium sulphate was treated the same. This yield is within $1\frac{1}{2}$ bushels of that of plot 13 which received the potassium sulphate. This beneficial effect produced by sodium sulphate is usually attributed to the liberation of potassium. While a considerable part of this beneficial effect is undoubtedly due to this cause, a study of the phosphorus yielded by each plot, will at least indicate that there is another factor entering. The average yield of phosphorus from plot 11 was 8.27 pounds (40) per acre; while the average on plot 12 was 9.82 pounds per acre. It may thus be seen that as an average of 20 years there were 1.55 pounds more of phosphorus taken from the sulphate plot than from the plot which received no sulphate. When one takes into consideration this excess of 31 pounds of phosphorus which had been removed it would seem that the sulphate had in some way made the phosphorus more available. As an average of 40 years the plot which received superphosphate alone had 16.46 per cent (41) phosphorus in the ash of the wheat. The plot which received sodium, potassium, and magnesium salts in addition to the superphosphate had 16.78 per cent phosphorus in the ash of the wheat.

The above facts point very strongly to a liberation of phosphorus by various sulphates. This is well shown by the work of

Dyer (42). He made a careful study of the soil from the Hoos Field, Rothamsted. The land had been in barley for 42 years. The plot which had received no manure was found to contain 22.27 pounds more per acre in the first nine inches than the one which had received ammonium salts. However, the amount soluble in a 1 per cent solution of citric acid was 5.24 pounds more in the latter than in the former. When the plots throughout the entire field were taken the same relationship was found to hold. There was more soluble phosphorus in every case in the plots which had received a sodium, potassium, magnesium, or ammonium salt. If we take Dyer's averages of the plots which were treated nearly alike this fact is brought out even more forcibly than the above. The four plots which received nitrogen, but no mineral manure yielded, as an average of 38 years, 28½ bushels of barley per acre. The soluble phosphorus in these plots was 69 pounds per acre. Now taking the four plots which received nitrogen, sodium, potassium and magnesium but no phosphoric acid, yielded as an average for the same length of time 30½ bushels, and contained 103.47 pounds per acre of soluble phosphorus. It may be seen that the latter in the course of 38 years yielded 72 bushels more barley than the former and at the end of this period had 32.61 pounds per acre more soluble phosphorus in the soil. Again, the four plots which received a complete fertilizer had an average yearly yield of 39⅜ bushels per acre. The plots which received only nitrogen and phosphorus yielded 38⅞ bushels. At the end of the period there were 549.7 pounds of soluble phosphorus in the one which received complete fertilizer; while the plot which received nitrogen and phosphorus only, had in the first nine inches 477.6 pounds of soluble phosphorus per acre. This is 72.1 pounds in favor of the plots which received sulphates.

Later Dyer (44) made a study of the Rothamsted wheat soil; determining the potassium and phosphorus present in a soluble and insoluble condition. The following is what he had to say concerning the effect of sodium, potassium, and magnesium salts on the solubility of phosphorus in the soil. The summary is quoted nearly in full on account of its direct bearing on the subject under consideration.

EFFECT OF ALKALINE SALTS ON THE SOLUBILITY OF PHOSPHORUS IN THE SOIL.

"It will have been seen that two of the six phosphate manured plots differ notably from the others in the subsoil contents of citric-acid-soluble phosphorus. These are plots 5 and 7. These two plots alone, in addition to phosphorus have had persistently supplied to them potassium, sodium, and magnesium salts (400 pounds per annum in the aggregate). To three of the other four plots one or the other of these salts has been supplied, but only to these two have all three salts been given. One of the two (plot 7) has received ammonium salts also in the same quantities as plots 11 to 14. The other (plot 5) has received the same full phosphatic and mineral saline dressing, but without ammonium salts.

Plot 5, getting no nitrogen, and yielding in consequence an annual average crop not very greatly exceeding that of the unmanured soil, has naturally accumulated a far larger quantity of phosphorus, nearly 218 pounds more per acre being found by analysis in the first 9 inches than in the average of the plots receiving both ammonium salts and mineral manures. As would be expected, there is also a much larger accumulation of citric-acid-soluble phosphorus and the proportion of citric-acid-soluble to total accumulation is greater than in the average of these other plots. Further, in both the second and third depths we find a tangible excess of citric-acid-soluble phosphorus beyond that in the unmanured plot, showing that the available phosphorus in the subsoil has been in excess of the demands of the crops. That this is not wholly due to the supply of a relatively large abundance of phosphorus without nitrogen, but also to another cause, appears on comparison with the results found in the case of plot 7, which in addition to a precisely similar liberal supply of phosphates and other saline minerals has received also ammonium salts.

Plot 7 has yielded in virtue of the full supply of potassium, sodium, and magnesium salts, in addition to phosphate and ammonium salts, a persistently larger yield of both wheat and straw than any of its companions. Consequently, its output of phosphorus has been greater and its accumulation less. Instead. however, of being poorer in available phosphorus it is now richer to the extent of some hundreds of pounds per acre than its companions which received either less alkaline salts or none.

It seems that the full supply of potassium, sodium, and magnesium salts has either exerted a solvent action on natural store of otherwise unavailable phosphorus in the soil, or, which appears more probable, that the manurial phosphates have entered into combination with the saline bases, and been retained in a less insoluble condition then where these have been absent or less in quantity.

Plot 11, which has received phosphates and ammonium salts only and has yielded smaller crops and accumulated more phosphorus than plots 12, 13, and 14, shows less citric-acid-soluble phosphorus than any of them. Next comes plot 12 which has annually received 366½ pounds per annum of sodium sulphate, and which, though accumulating less phosphorus, is

nevertheless appreciably richer in citric-acid-soluble. Next is plot 14, accumulating almost the same phosphorus as plot 12, but getting 280 pounds per annum of magnesium sulphate, and showing, in the surface soil, 32.75 pounds more of citric-acid-soluble phosphorus though less in the subsoil. Next come plots 13 and 14, getting respectively 200 pounds of potassium sulphate and 280 pounds of magnesium sulphate per annum, There have accumulated respectively 27.94 pounds less and 5.24 pounds more of phosphorus per acre than plot 12, but are respectively 24 pounds and 32.75 pounds richer in citric-acid-soluble in the first 9 inches.

The following figures show the proportion borne by the excess of citric-acid-soluble phosphoric acid over plot 3 (both in the first 9 inches and in the whole 27 inches) to the calculated accumulated excess over plot 3.

1893 SAMPLES.

Ratio of excess of citric-acid-soluble phosphoric acid over unmanured plot to calculated excess of phosphoric acid supplied per acre over unmanured plot, the calculated excess in each case being taken as 100.

PLOT.	TREATMENT.	FIRST 9 INCHES.	27 INCHES.
		per cent.	*per cent.*
5	Phosphates, potassium, sodium and magnesium salts	48	50
7	Ammonium salts, phosphates, potassium, sodium and magnesium salts	50	50
13	Ammonium salts, phosphates and potassium salts	36	34
14	Ammonium salts, phosphates and magnesium salts	35	34
12	Ammonium salts, phosphates and sodium salts	33	32
11	Ammonium salts, and phosphates only	31	29

It appears that the action of sodium sulphate (366 pounds per annum) in maintaining the phosphate in an easy soluble condition has been somewhat less than that of magnesium sulphate (280 pounds per annum), and that this again has had less effect than potassium sulphate (200 pounds per annum). But the addition to the 200 pounds of potassium sulphate of 100 pounds magnesium sulphate and 100 pounds sodium sulphate has greatly affected the condition of the phosphorus in the surface soil, and has appreciably affected it in the subsoil."

The above facts tend to show that the solvent action of potassium was greater than that of sodium or magnesium salts. However, when we take into consideration the fact that sodium and magnesium sulphate carry considerable water of crystallization,

we find there was more of the dry potassium sulphate applied than either of the other salts. There would be of the dry salts 200 pounds of potassium sulphate, 162 pounds of sodium sulphate, and 137 pounds of magnesium sulphate. The relationship is brought out clearly in the following table which shows the ratio between the dry salt applied and the soluble phosphorus found in the soil.

PLOT.	TREATMENT.	FIRST 9 INCHES.	FIRST 27 INCHES.
12	Ammonium salts, phosphates and sodium salts..................................	10.6	10.4
13	Ammonium salts, phosphates and potassium salts..................................	9.2	8.7
14	Ammonium salts, phosphates and magnesium salts..............................	13.8	13.2

From the above it may be seen that pound for pound the magnesium salts are the most effective in keeping the phosphorus soluble. The sodium sulphate comes next and the potassium salts have least effect.

All the above facts point to the conclusion that some soluble salts when applied to the soil either alone or in connection with a phosphate tend to made the phosphate more available to the plant. The salts which appear to have this effect are calcium, iron, sodium, ammonium, potassium, and magnesium sulphate and ammonium nitrate. This may be due to either of the following causes or both. First, to the feeding power of the plant, or in the case of the ammonium compounds to bacteria, which break down the salt applied and render the soil solution slightly acid. That this may in part account for the solvent action is indicated by the effect of ammonium sulphate on soil. When this compound is used for some time on a land poor in lime the land becomes acid. Now even though this acidity be ever so small, with most salts on ordinary soil, there would still be its action on soil particles which would tend to make the plant food more available. Second, this effect may be due to the direct solvent action of the salt as applied or indirectly by its reaction with salts already in the soil and their subsequent action on the

phosphates. Considerable light will be thrown on this solvent action by means of the laboratory tests which have been carried on in connection with the above study.

EFFECT OF SALINE SOLUTIONS ON PHOSPHATES.

Historical.

A number of experimenters have done some work, in the laboratory, to determine the solvent action of various salts in solution on phosphates. This, in the main, has been fragmentary, one making a few tests on one compound under certain conditions, while others working with different compounds and maybe under different conditions made other tests. Again, many of these tests were made on chemically pure substances. While this may give us the action of the solvent on the phosphate, it will not, however, give us the indirect action if there be one. That is, there may be a reaction between certain compounds in a soil or impure phosphate, and then an interaction between this new compound and some element in the phosphate. Or it may be that the solution of the new compound formed will hold more of the phosphate in solution than the original solution would. Some of the experimenters have based their conclusions on single tests; another test may reverse the conclusion. Again, some of the work was done before the present delicate methods for determining phosphorus were known.

The following is a brief summary of the most important work done on this subject.

Schulov (45) found that a solution of ammonium sulphate extracted more phosphorus from a phosphorite than did the same volume of distilled water. This was found to be true with the nitrate also, but not to as great an extent as with the sulphate solution.

Cameron (46), working with chemically pure iron phosphate, aluminum phosphate, and calcium phosphate, found that the calcium phosphate was slightly more soluble in a solution of potassium chloride, and less soluble in calcium chloride and calcium nitrate than in distilled water. The iron phosphate was more soluble in a potassium sulphate solution and less soluble in a potassium chloride and a sodium nitrate solution than in water. He

showed further that equilibrium was not established until at least ten days after the solution had been added to the phosphate.

Kalmann (47), extracted soil with a solution of calcium sulphate and with distilled water and obtained the same amount of phosphorus in each case.

Feedler (48), obtained less phosphorus by extracting the soil with sodium nitrate solution than with distilled water. Krouch (49), however, obtained more with a sodium chloride solution than with water. Thompson (50), found the same to hold true when a superphosphate was used in the place of soil.

Both Kellner (51), and Sutherst (52), found that lime rendered the phosphorus of the soil more soluble. Later, Hartwell (53), and Kellogg at the Rhode Island Station found the same to be true.

Voelcker (54), working with phosphate, bone meal, Cambridge and Suffolk caprolites, found that they were all more soluble in ammonium chloride and in ammonium carbonate solutions than in distilled water. Sodium nitrate solution extracted more of the phosphate than did water while the caprolites yielded the same to each solvent.

Sutherst (52), found that lime extracted more phosphorus from iron and aluminum phosphates than water but when calcium carbonate was used in place of the lime the same amount of phosphorus was obtained with each solvent. He also found that potassium chloride and sodium chloride solutions each extracted less from bone meal than did distilled water. However, when he used bone flour in place of bone meal each salt extracted considerable more than the distilled water. He explains this apparent contradiction by assuming that bone meal, when in the soil, undergoes fermentation by which the phosphorus is rendered more soluble but when quantities of inorganic salts are present this fermentation is prevented.

Liebig (57), showed that sodium nitrate increased the solubility of calcium phosphate, while Lachonicy (58), found that it decreased the solubility of iron phosphate.

It may be seen from the above summary that lime, the chloride, carbonate, sulphate, and nitrate of ammonia, were always found to increase the solubility of a phosphate. The nitrate, chloride, and carbonate of calcium were found to always decrease the solu-

bility of phosphates while the sulphate had no effect. Potassium sulphate rendered the iron and aluminum phosphates less soluble but it increased the solubility of calcium phosphate. Sodium nitrate with two exceptions extracted less than water. And it is to be noticed that in these cases the compounds were bone meal, which is rich in calcium phosphate, and a chemically pure calcium phosphate. So it would seem as if the calcium phosphate is more soluble and the iron phosphate less soluble in sodium nitrate solutions than in distilled water. The results obtained where sodium chloride was used as the solvent vary and with the data at hand it is not easy to explain this difference.

EXPERIMENTAL.

On account of the great importance of the phosphates in agriculture, and especially the insoluble ones, as they are the ones which would be found in largest quantities in the soil, it seems that time would not be badly spent in making a test of the solvent action of various salts on insoluble phosphates, to see if more definite information can be obtained on this subject.

In making this study six phosphates, three brown, two blue, one white, and a combination of brown phosphate and soil, have been extracted with solutions of the following compounds; sodium, potassium, calcium, magnesium, ammonium and ferrous sulphate, also the chlorides and nitrates of sodium, potassium, calcium, magnesium, and ammonium.

Methods of Investigation.

Two grams of the phosphate to be tested were treated in a 1000cc. glass stoppered bottle with 500 cc. of a[1] 1 per cent solution of each of the above compounds. This was let stand with occasional shaking for from 10 to 14 days. At the end of this time they were filtered through 18 cm. filter paper under which was a 11 cm. filter. To 450 cc. of the solution thus obtained were added 10 cc. of a 1 per cent solution of iron chloride, the solution acidified and evaporated nearly to dryness. While still hot this was precipitated

[1]In the case of calcium sulphate a saturated solution of the salt was used.

with ammonium hydroxide, care being taken not to get an excess, and filtered, and washed with hot water. The precipitate was dissolved with nitric acid, evaporated to dryness, taken up with a few drops of hydrochloric acid, evaporated to dryness and heated in an air oven for 30 minutes at 105° C. This residue was taken up with acidulated water, the silica filtered off and washed with hot water and the filtrate concentrated to 10 or 20 cc. This was treated with ammonium hydroxide and barely enough nitric acid added to dissolve the precipitate which was formed. The solution was then heated to 56° C. and 10 cc. of molybdic acid solution of the same temperature added, and kept at this temperature for two hours. At the end of this time it was set in a cool place for about twelve hours. The solution was then filtered and washed with a .1 per cent solution of ammonium nitrate until free of acid. the last two washings being with cold distilled water. The precipitate was dissolved in potassium hydroxide of such a strength that 1 cc. of the solution was equivalent to .2 mg. of phosphorus. The excess of alkali was titrated with a nitric acid solution 1 cc. of which was equivalent to 1cc. of the standard alkali, using phenolphthalein as an indicator.

Dark Brown Rock Phosphate.

The first sample tested was a dark brown phosphate containing 10.6 per cent of the element phosphorus. This was a sample of the phosphate used by the Illinois Agricultural Experiment Station. It was taken just as used on the soil, i. e., without further grinding.

TABLE 1

Phosphorus dissolved by 450 cc. of 1 per cent solution of each of the following solvents from 2 grams of dark brown rock phosphate.

SOLVENT USED.	PHOSPHORUS IN MILLIGRAMS.						
	1	2	3	4	5	Average.	Difference from that extracted by water.
Distilled water	0.58	0.59	0.36	0.36	0.21	0.42	
Ammonium nitrate ..	0.86	0.90	0.61			0.79	0.37
Ammonium sulphate.	0.86	0.82	0.54	0.56	0.58	0.67	0.25
Sodium sulphate.....	0.86	0.90	0.78	0.82	0.66	0.80	0.38
Ammonium chloride.	0.94	0.98	0.56	0.59	0.62	0.74	0.32
Potassium sulphate..	0.68	0.72	0.74	0.78	0.65	0.71	0.29
Magnesium sulphate.	0.74	0.80	0.60	0.86	0.42	0.68	0.26
Magnesium nitrate..	0.52	0.48	0.42	0.38		0.45	0.03
Sodium nitrate	0.58	0.59	0.28	0.26		0.43	0.01
Magnesium chloride.	0.62	0.57	0.48	0.58	0.48	0.55	0.13
Potassium chloride..	0.58	0.63	0.30	0.30	0.40	0.44	0.02
Potassium nitrate...	0.50	0.64	0.49	0.46		0.52	0.10
Sodium chloride......	0.54	0.52	0.50	0.42	0.32	0.46	0.04
Calcium nitrate......	0.24	0.24	0.36	0.52		0.34	0.08
Gypsum.............	0.22	0.20				0.21	—0.21
Calcium[1] sulph. C. P.	0.20	0.26				0.23	—0.19
Calcium chloride.....	0.14	0.16	0.12	0.16	0.10	0.13	—0.29
Iron sulphate........	0.20	0.22	0.14	0.12	0.24	0.18	—0.24
Distilled water.......	0.58	0.59	0.36	0.36	0.21	0.42	

[1] The first solution of calcium sulphate, marked C. P., used extracted from 2 to 5 mg. of P, but on testing it was found to be slightly acid.

It may be seen from Table 1 that the duplicate tests in the main agree fairly well. Columns no. 1 and 2 do not fully agree with columns 3 and 4 but this difference is very likely due to a difference of temperature and the amount of shaking received during extraction. However, the same relationship exists throughout between the amount extracted by water and the various solvents. In every case the calcium and iron solutions extracted less phosphorus than the water. Sodium, magnesium, ammonium, and potassium sulphate, magnesium and ammonium chloride, and ammonium and potassium nitrate extracted considerably more phosphorus than distilled water. This is especially high in the case of sodium sulphate and the ammonium compounds. The amount extracted by sodium and potassium chlo-

ride and sodium and magnesium nitrate is about the same as that extracted by distilled water. The table shows very plainly that the sulphates in every case extracted more than the nitrates and they in turn extracted more than the chlorides.

Phosphate and Soil.

The same phosphate was tested with soil to see if it would change the solubility. In this case eight grams of a sandy loam soil was mixed with two grams of the phosphate and extracted as in the previous case.

TABLE 2

Phosphorus dissolved by 450 cc. of a 1 per cent solution of each of the following solvents, from 2 grams of phosphate and 8 grams of soil.

SOLVENT USED.	PHOSPHORUS IN MILLIGRAMS.			
	1	2	Average.	Difference from that extracted by water.
Distilled water	0.76	0.78	0.77	
Ammonium nitrate	1.44	1.46	1.45	0.68
Ammonium sulphate	0.80	0.84	0.82	0.05
Sodium sulphate	0.96	0.98	0.97	0.20
Ammonium chloride	0.58	0.60	0.59	−0.18
Potassium sulphate	0.88	0.86	0.87	0.10
Magnesium sulphate	0.68	0.70	0.69	−0.08
Magnesium nitrate	1.24	1.22	1.23	0.46
Sodium nitrate	0.96	0.95	0.96	0.18
Magnesium chloride	0.70	0.66	0.68	−0.09
Potassium chloride	0.44	0.42	0.43	−0.34
Potassium nitrate	0.86	0.84	0.85	0.08
Sodium chloride	0.80	0.74	0.77	0.00
Calcium nitrate	0.44	0.46	0.45	−0.32
Calcium sulphate C. P	0.39	0.42	0.40	−0.37
Calcium chloride	0.40	0.44	0.42	−0.35
Iron sulphate	0.32	0.28	0.30	−0.40
Distilled water	0.76	0.78	0.77	

Eight grams of the soil alone was extracted with each of the solvents. The calcium and iron compounds did not extract enough phosphorus from the soil to give qualitative tests while the rest of the solvents extracted barely traces. Therefore, practically all of the phosphorus reported in table 2 is from the phosphate.

The noteworthy facts shown in table 2 are as follows. The amount of phosphorus extracted by each solvent is much greater where soil is used with the phosphate than where either is used alone, being especially marked in the case of the nitrates. Where the soil is used with the phosphate the nitrates extract more phosphorus than the sulphates, but where the phosphate is extracted alone the reverse is true. When soil is mixed with the phosphate the calcium and iron compounds and all the chlorides extract less than distilled water. Sodium and potassium sulphate, magnesium and ammonium nitrate extract more than water.

Light Brown Rock Phosphate.

A sample of light brown phosphate of loose texture containing 12.6 per cent of phosphorus, was ground and extracted with each of the solvents. The grinding was done in a large steel mortar, and may not have been ground as fine as the preceding sample. This may account for the small amount of phosphorus extracted by each solvent.

TABLE 3

Phosphorus dissolved by 450 cc. of a 1 per cent solution of each of the following solvents, from two grams of light brown rock phosphate.

SOLVENT USED.	PHOSPHORUS IN MILLIGRAMS.					
					Average.	Difference from that extracted by water.
Distilled water	0.30	0.23	0.36	0.42	0.33	
Ammonium nitrate	1.70	0.78	1.56	1.66	1.43	1.10
Ammonium sulphate	0.38	0.36	0.48		0.41	0.08
Sodium sulphate	0.27	0.20	0.36	0.36	0.30	– .03
Ammonium chloride	0.33	0.26	0.56	0.58	0.43	0.10
Potassium sulphate	0.34	0.38	0.36	0.36	0.36	0.03
Magnesium sulphate	0.34	0.34	0.46	0.34	0.37	0.04
Magnesium nitrate	0.50	0.32	1.18	0.62	0.66	0.33
Sodium nitrate	0.32	0.20	0.34	0.36	0.31	–0.02
Magnesium chloride	0.30	0.28	0.22	0.26	0.26	–0.07
Potassium chloride	0.20	0.22	0.18	0.30	0.23	–0.10
Potassium nitrate	0.29	0.23	0.26	0.36	0.28	–0.05
Sodium chloride	0.18	0.36	0.26	0.14	0.21	–0.12
Calcium nitrate	0.36	0.38	0.14		0.37	0.04
Gypsum	0.20	0.22	0.37		0.21	–0.12
Calcium sulphate C. P	0.18	0.14			0.16	–0.17
Calcium chloride	0.20	0.18	0.18	0.12	0.17	–0.16
Iron sulphate	0.15	0.11	0.18	0.20	0.16	–0.17
Distilled water	0.30	0.23	0.36	0.42	0.33	

An examination of table 3 shows that to a certain extent there is the same regularity as observed before. The calcium and iron compounds with the exception of calcium nitrate have prevented the phosphorus from going into solution. The sodium, potassium, and magnesium chlorides seem to have exerted this influence also. The ammonium compounds and magnesium nitrate are the only ones which have, to any great extent, exerted a solvent action on the phosphate. The amount extracted by ammonium nitrate was very large being nearly five times as great as that extracted by water. Columns 3 and 4 were extracted for fourteen days, and they are almost invariably higher than columns 1 and 2 which were extracted but ten days. This would indicate that equilibrium was not established at the end of ten days.

White Rock Phosphate.

The next phosphate tested contained 13.3 per cent of the element phosphorus. It was much harder than the phosphates previously tested and was nearer a pure calcium phosphate containing but little iron. The phosphate was ground in an agate mortar until free from grit then extracted with the various solvents.

TABLE 4

Phosphorus dissolved by 450 cc. of a 1 per cent solution of each of the following solvents, from 2 grams of white rock phosphate.

SOLVENT USED.	PHOSPHORUS IN MILLIGRAMS.			
	1	2	Average.	Difference from that extracted by water.
Distilled water	0.34	0.40	0.37	
Ammonium nitrate	1.14	1.18	1.16	0.79
Ammonium sulphate	0.90	0.96	0.93	0.56
Sodium sulphate	0.86	0.78	0.82	0.45
Ammonium chloride	0.74	0.96	0.85	0.48
Potassium sulphate	0.68	0.78	0.73	0.36
Magnesium sulphate	0.66	0.72	0.69	0.32
Magnesium nitrate	0.82	0.68	0.70	0.33
Sodium nitrate	0.82	0.74	0.78	0.41
Magnesium chloride	0.60	0.64	0.62	0.25
Potassium chloride	0.62	0.50	0.56	0.17
Potassium nitrate	0.66	0.64	0.65	0.28
Sodium chloride	0.34	0.44	0.39	0.02
Calcium nitrate	0.14	0.16	0.15	−0.22
Gypsum	0.18	0.26	0.22	−0.15
Calcium sulphate C. P.	0.22	0.23	0.22	−0.15
Calcium chloride	0.14	0.24	0.19	−0.18
Iron sulphate	0.12	0.16	0.14	−0.23
		0.36		
Distilled water	0.34	0.40	0.37	

The calcium and iron compounds extract less phosphorus than distilled water while the sodium extracts about the same as distilled water. All the remaining compounds render the phosphate more soluble. This is especially marked with the nitrates and sulphates of sodium and potassium, and all the ammonium compounds.

Blue Rock Phosphate.

The next sample tested was a blue phosphate, the outer edges of which had weathered and had become dark brown. The sample was separated into two parts, weathered and unweathered. The former contained 9.9 per cent phosphorus and was harder than the latter, which contained but 8.9 per cent of phosphorus. The samples were tested separately with the results reported in table 5.

TABLE 5

SOLVENT USED.	UNWEATHERED PHOSPHATE. PHOSPHORUS IN MG.				WEATHERED PHOSPHATE. PHOSPHORUS IN MG.			
	1	2	Average.	Diff. from distilled water.	1	2	Average.	Diff. from distilled water.
Distilled water......	0.20	0.26	0.23		0.39	0.41	0.40	
Ammonium nitrate..	0.54	0.52	0.53	0.30	0.54	0.78	0.66	0.26
Ammonium sulphate	0.26	0.26	0.26	0.03	0.72	0.74	0.73	0.33
Sodium sulphate.....	0.16	0.18	0.17	−0.06	0.56	0.52	0.54	0.14
Ammonium chloride.	0.28	0.42	0.35	0.12	0.40	0.48	0.44	0.04
Potassium sulphate..	0.22	0.28	0.25	0.02	0.51	0.49	0.50	0.10
Magnesium sulphate.	0.28	0.28	0.28	0.05	0.49	0.46	0.48	0.08
Magnesium nitrate..	0.36	0.30	0.33	0.10	0.32	0.28	0.30	−0.10
Sodium nitrate......	0.16	0.24	0.20	−0.03	0.36	0.36	0.36	−0.04
Magnesium chloride.	0.29	0.22	0.25	0.02	0.36	0.22	0.29	−0.11
Potassium chloride..	0.16	0.14	0.15	−0.08	0.36	0.42	0.39	−0.01
Potassium nitrate...	0.15	0.18	0.16	−0.07	0.27	0.33	0.30	−0.10
Sodium chloride.....	0.18	0.14	0.16	−0.07	0.32	0.28	0.30	−0.10
Calcium nitrate......	0.24	0.20	0.22	−0.01	0.20	0.15	0.17	−0.23
Gypsum	0.16	0.18	0.17	−0.06	0.14	0.16	0.15	−0.25
Calcium sulphate C. P..................	0.14	0.22	0.18	−0.05	0.18	0.20	0.19	−0.21
Calcium chloride....	0.08	0.12	0.10	−0.13	0.04	0.16	0.10	−0.30
Iron sulphate.......	0.02	0.06	0.04	−0.19	0.06	0.08	0.07	−0.33
Distilled water	0.20	0.26	0.23		0.39	0.41	0.40	

The weathered phosphate yielded its phosphorus to solvents much more readily than the unweathereed. With the exceptions of sodium sulphate, magnesium chloride and nitrate the same relationship is found in both phosphates. The calcium and iron

compounds, sodium and potassium chloride and nitrate prevented the phosphorus from going into solution. The ammonium compounds, as usual, had the greatest solvent action.

Another sample of blue phosphate containing 8.5 per cent of phosphorus was tested. This yielded enough phosphorus to the magnesium and ammonium sulphate, ammonium chloride and ammonium and potassium nitrate solutions to give a qualitative test. None of the other solvents extracted sufficient for a test. So it may be seen that the solvents which extracted a comparatively large amount of the preceding phosphate gave a qualitative test with the second.

Summary of Solubility Tests.

The relationship existing between the various phosphates and solvents is brought out much clearer if the results with like phosphates are brought together. The light brown, dark brown, and weathered blue can very naturally be classed together as weathered phosphate. The white, while equally a weathered phosphate, seems to have been worked over and differs from the brown in being nearer pure calcium phosphate. The two samples of blue can be classed as unweathered.

The following table shows the relationship existing between the various weathered and unweathered phosphates stated as mg. of phosphorus extracted from two grams of each phosphate by 450 cc. of the various solvents.

TABLE 6

Phosphorus dissolved by 450 cc. of a 1 per cent solution of each of the following solvents, from 2 grams of each of the phosphates. Average of all the determinations.

SOLVENT USED.	WEATHERED PHOSPHATE.				UNWEATHERED PHOSPHATE.	
	PHOSPHORUS IN MG.				PHOSPHORUS IN MG.	
	Light Brown.	Dark Brown.	Weathered Blue.	Average.	No. 1 Blue.	No. 2 Blue.
Distilled water.........	0.33	0.44	0.40	0.37	0.23	
Ammonium nitrate.....	1.43	0.79	0.66	0.96	0.53	traces
Ammonium sulphate...	0.41	0.67	0.73	0.60	0.26	traces
Sodium sulphate.......	0.30	0.80	0.54	0.55	0.17	
Ammonium chloride...	0.43	0.74	0.44	0.53	0.35	traces
Potassium sulphate....	0.36	0.66	0.50	0.51	0.25	
Magnesium sulphate...	0.37	0.68	0.48	0.51	0.28	traces
Magnesium nitrate.....	0.66	0.45	0.30	0.47	0.33	
Sodium nitrate.........	0.31	0.43	0.36	0.37	0.20	
Magnesium chloride....	0.26	0.57	0.29	0.37	0.25	
Potassium chloride.....	0.23	0.44	0.39	0.35	0.15	
Potassium nitrate......	0.28	0.48	0.30	0.35	0.16	traces
Sodium chloride........	0.21	0.46	0.30	0.33	0.16	
Calcium nitrate........	0.37	0.39	0.17	0.28	0.22	
Gypsum...............	0.21	0.21	0.18	0.20	0.20	
Calcium sulphate C. P .	0.16	0.23	0.16	0.18	0.15	
Calcium chloride.......	0.17	0.13	0.10	0.13	0.10	
Iron sulphate...........	0.17	0.18	0.07	0.14	0.04	
Distilled water.........	0.33	0.44	0.40	0.37	0.23	

The above table shows that the sodium, potassium and magnesium sulphates and all the ammonium compounds extract more phosphorus from the weathered phosphate than is extracted by the same volume of distilled water. All the calcium and iron compounds and sodium nitrate depress the solubility of the phosphate. The remaining solvents have little effect or else the effect varies with the phosphate. The most phosphorus is extracted from the unweathered phosphate by the ammonium compounds, magnesium sulphate, and potassium nitrate.

Inasmuch as there is a similarity in all the weathered phosphates we can take an average of them and compare this average with the average of the unweathered, white, and a mixture of soil and

brown. In this way factors that are common to all are brought out. Furthermore, it shows the effect of soil on brown phosphate.

In studying table 7 we find that with all the phosphates the calcium compounds have depressed their solubility. This is what may have been expected for the phosphate and calcium salt would each form common ions (Ca) and if the two solutions, the calcium phosphate solution and the other calcium salt solution, were brought together the calcium ion from the calcium salt would force back, as it were, some of the calcium ions from the phosphate with the result that more of the phosphate would be precipitated; hence we would find less soluble phosphorus in the calcium salt solution than in water.

TABLE 7

Average of phosphorus dissolved by 450 cc. of a 1 per cent solution of each of the following solvents from 2 grams of each of the phosphates.

SOLVENT USED.	PHOSPHORUS IN MILLIGRAMS.			
	Weathered Phosphate.	Unweathered Phosphate.	White Phosphate.	Soil and Phosphate.
Distilled water	0.37	0.23	0.37	0.77
Ammonium nitrate	0.96	0.53	1.16	1.45
Ammonium sulphate	0.60	0.26	0.93	0.82
Sodium sulphate	0.55	0.17	0.82	0.97
Ammonium chloride	0.53	0.35	0.85	0.59
Potassium sulphate	0.51	0.25	0.73	0.95
Magnesium sulphate	0.51	0.28	0.69	0.69
Magnesium nitrate	0.47	0.33	0.70	1.25
Sodium nitrate	0.37	0.20	0.78	0.96
Magnesium chloride	0.37	0.25	0.62	0.68
Potassium chloride	0.35	0.15	0.56	0.43
Potassium nitrate	0.35	0.16	0.65	0.81
Sodium chloride	0.32	0.16	0.39	0.77
Calcium nitrate	0.28	0.22	0.15	0.45
Gypsum	0.20	0.20	0.25	
Calcium sulphate C. P	0.18	0.15	0.20	0.40
Calcium chloride	0.13	0.10	0.19	0.42
Iron sulphate	0.14	0.04	0.14	0.30
Distilled water	0.37	0.23	0.37	0.77

In every case the iron solution extracted less than the water. This would also appear reasonable, for if there be any change in the basic ions there would be formed iron phosphate which is still

less soluble in water than calcium phosphate. In every case the potassium sulphate, ammonium sulphate, magnesium and ammonium nitrate extracted more phosphorus than did water. Disregarding the result obtained with the soil and phosphate mixed, we find that magnesium sulphate and ammonium chloride rendered the phosphate more soluble. This, however, is reversed on addition of soil to the phosphate. Sodium sulphate, except with the unweathered phosphate, extracted more than water. Comparing the results with sodium and potassium nitrate we find where the brown phosphate was used these solvents depressed the solubility, where the white phosphate (a phosphate containing more calcium and little iron) was used they rendered the phosphate more soluble. This is in accord with the work of other experimenters who have found that these salts depress the solubility of iron phosphate and increase the solubility of calcium phosphate. Sodium, magnesium, and potassium chloride have little if any effect on the phosphate.

Throughout the work it has been noted that ammonium sulphate and especially ammonium nitrate rendered the phosphate more soluble. This was thought to be due to nitrification but on testing for nitrates by means of the Gill's method there was found to be no more nitrates present at the end than at the beginning of extraction.

SUMMARY AND CONCLUSIONS.

A number of eminent writers have noted the fact that some salts render insoluble phosphates more soluble.

Sodium nitrate and ammonium nitrate give better yields than an equivalent amount of nitrogen in the form of dried blood.

Ammonium nitrate and sulphate are the best form of nitrogen to use in connection with insoluble phosphates.

Ammonium salts, especially ammonium nitrate increase the phosphorus of plants.

A number of experimenters have obtained good results from the use of common salt in connection with insoluble phosphates.

It seems to be well established that lime acts as a solvent on phosphates.

Some experimenters have found that iron sulphate increases the phosphorus of plants.

The Rothamsted experiments point to the conclusion that potassium, sodium, magnesium and ammonium sulphate liberate phosphorus.

Dyer's analysis of the Rothamsted soil showed that the phosphorus was more "available" in the soil which had been treated with sodium, potassium, magnesium and ammonium sulphate.

Various experimenters have shown that lime, ammonium nitrate, ammonium sulphate, ammonium carbonate and ammonium chloride increase the solubility of phosphates.

Sodium nitrate exerts a solvent action on calcium phosphate and depresses the solubility of iron phosphate.

Further investigation showed the following:

Calcium and iron salts render phosphates less soluble.

Sodium sulphate, calcium sulphate, ammonium sulphate, ammonium chloride, ammonium nitrate, and magnesium nitrate render phosphates more soluble.

Sodium and potassium nitrate render calcium poshphate more soluble and iron phosphate less soluble.

The effect of magnesium sulphate, sodium chloride, magnesium chloride and potassium chloride is small or it varies with the different phosphates.

The presence of soil with the phosphate causes the solvent to act more vigorously; *especially is this the case* with ammonium nitrate.

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THE DETERMINATION OF SMALL QUANTITIES OF IODINE, WITH SPECIAL REFERENCE TO THE IODINE CONTENT OF THE THYROID GLAND.

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In the course of an investigation which was carried out in collaboration with Simpson, and which is being reported elsewhere,[1] I had occasion to make a series of iodine estimations on the thyroid glands of sheep, and undertook also the still more delicate task of measuring the traces of that substance which may occur in the pituitary.[2] Practically the only method that has been applied to such determinations is the original one of Baumann,[3] in one or other of its several modifications. It is a method, which in practised hands yields undoubtedly excellent results. Nevertheless the very multiplicity of the improvements it has suffered is sufficient indication that more or less dissatisfaction has from time to time been felt with it.[4] This dissatisfaction I came, after some preliminary trials, to share; and was led accordingly to seek some other mode of iodine estimation, which should be applicable to the problem. Since the method finally adopted has proved in my hands to be both convenient and accurate, and to possess in these respects some points of advantage over the older one, I believe myself justified in describing it now in detail;[5] the more so, as much importance

[1] Simpson and Hunter: *Quart. Journ. of Physiol.*, iii, 1910.

[2] See Wells: *Journ. Amer. Med. Ass.*, Oct. 30, Nov. 6 and 13, 1897; Schnitzler: *Wien. med. Wochenschr.*, 1896, p. 657.

[3] Baumann and Roos: *Zeitschr. f. physiol. Chem.*, xxi, p. 489.

[4] For the latest criticism see Riggs: *Journ. Amer. Chem. Soc.*, xxxi, p. 711.

[5] It has been already communicated in preliminary form to the Soc. for Exp. Med. and Biol. See *Proceedings*, vii, p. 10. 1909.

attaches at present to exact knowledge of the iodine content of given specimens of thyroid.

Baumann's method, whether in its original or its later forms, has two separate stages. The first strives to effect, simultaneously with the destruction of the organic matter, the quantitative conversion of the iodine into an alkaline *iodide;* in the second the iodine liberated from this combination is estimated by colorimetry. The method now to be described and discussed proceeds by two corresponding steps; but the object it is sought to attain is the complete transformation of the iodine to the *iodic* state; and the final measurement is titrimetric.

FIRST STAGE OF THE PROCESS—COMBUSTION OF THE ORGANIC MATTER WITH FIXATION OF THE IODINE.

In the Baumann process this is accomplished by fusion with caustic soda, and subsequent oxidation of the char with potassium nitrate. Anyone, who has had occasion to fuse animal tissues with caustic soda, will I think admit that the operation is both tedious and disagreeable. Owing partly to the large amount of water unavoidably present, sometimes indeed purposely added, the melting mass has a strong tendency to froth, and is liable at any moment, unless extreme care is exercised, to be carried by escaping steam and gases over the edge of the crucible. The fusion must therefore be carried out with excessive slowness, and it requires constant vigilance on the part of the operator to prevent serious loss of material. The simultaneous performance by this method of any considerable number of analyses is therefore rendered exceedingly risky; while, to add to the unpleasantness of the whole, the gaseous products of the combustion are offensive in odor, and exceedingly apt to catch fire.

Apart from these merely manipulative difficulties, the main importance of which lies after all in the time consumed in overcoming them, it would appear to be at least open to question whether the object of the procedure—the quantitative conversion of the iodine into sodium *iodide*—is always really attained. The complete combustion of the charred mass by potassium nitrate might seem to introduce the risk of raising part of the iodine to the

iodic state. If more than one sixth of the total iodine should thus suffer oxidation, the excess would be lost to the subsequent determination. Baumann prescribed special precautions to prevent such an occurrence; but Riggs[1] has claimed recently to show that even if these are scrupulously observed, we have here a really important source of error. It must be admitted that there are difficulties in accepting unreservedly the conclusions drawn by Riggs from his experiments; and Seidell,[2] contending that a formation of iodate is easily excluded, attributes his results entirely to insufficient extraction.[3] The point remains in dispute, and, while it does so, the *possibility* at least of oxidation of the iodide has to be reckoned with.

The inconveniences and the uncertainty of the Baumann fusion are almost entirely eliminated by substituting for caustic alkali an anhydrous alkaline carbonate, and by choosing for the subsequent estimation of the iodine a method in which its partial oxidation during fusion would be no disadvantage.

A mixture of sodium carbonate with saltpeter is the vehicle employed for the destruction of organic matter in the familiar Volhard[4] process for halogen estimation, It was applied with success by Hofmeister[5] to the analysis of iodised proteins. There is no apparent reason why the same mixture should not prove equally well suited to the present problem. The proportion of potassium nitrate prescribed by Volhard is, however, greater than is actually required, and I have found it advisable to use less. Further, as has been pointed out by Neumann and Meinertz,[6] the double carbonate of sodium and potassium (or, what comes to the same thing, a mixture of the simple carbonates in equimolecular proportions) possesses certain advantages over soda by itself. Compounds of potassium are in general more energetic reagents than the corresponding compounds of sodium;

[1] Riggs: *loc. cit.*

[2] Seidell: *Journ. Amer. Chem. Soc.*, xxxi, p. 1326.

[3] We should then be driven by Riggs's data to the equally disturbing conclusion, that the degree of extraction actually attained, under conditions as nearly uniform as it is possible to make them, may be a very variable quantity.

[4] Volhard: *Annalen d. Chem.*, cxc, p. 40

[5] Hofmeister, *Zeitschr. f. physiol. Chem.*, xxiv, p. 163.

[6] Neumann and Meinertz: *Zeitschr. f. physiol. Chem.*, xliii, p. 37.

while the mixture has the additional convenience of melting at a lower point than either of its components. Neumann and Meinertz recommended the double carbonate originally for sulphur determinations, and they combined it with sodium peroxide; its use along with potassium nitrate in the estimation of iodine has proved equally satisfactory. The following are the proportions of the ingredients found to be most suitable:

	GRAMS.
Anhydrous potassium carbonate	138 (1 mol.)
Anhydrous sodium carbonate	106 (1 mol.)
Potassium nitrate	75 (¾ mol.)

These should be thoroughly powdered, intimately mixed, and kept in a well stoppered bottle.

Details of the combustion process. Into a nickel crucible of 125 cc. capacity is weighed about 1 gm. (correct to within 5 mgm.) of the thoroughly dry and finely powdered material to be analysed. Fifteen to twenty grams of the above oxidation mixture are added, and the whole is *intimately* mixed with the aid of a clean, dry, nickel spatula. Any particles that may adhere to the spatula are brushed back into the crucible, and 3 to 5 grams more of the mixture are spread evenly upon the surface. The lid of the crucible is laid on in such a way as to leave an open chink for the escape of gases. Combustion is then proceeded with.

In most of the methods that involve the use of such an oxidising mixture, one is recommended to proceed very slowly and cautiously with the application of heat. In the present case it is not necessary to do so. The crucible is to be heated strongly from the beginning. The best results are obtained by placing it at just such a height in the flame of a medium-sized Bunsen that it is enveloped to the level of its contents. These are thus brought almost immediately to a not too bright red heat, at which oxidation proceeds both rapidly and quietly. If the burning material has been finely enough divided and sufficiently intimately mixed (and these must be insisted on as essential points of the procedure) there is no violent deflagration or sputtering. On raising the lid of the crucible one can observe carbonisation and incineration to follow each other steadily from the edge to the center of the mass. As soon as the whole is seen to be uniformly white, the flame is removed. By this time the edge has usually just begun to melt.

Carried out in the way described the combustion is over in ten minutes or less. This implies, in a comparison with the Baumann process, a very considerable saving of time. Moreover, since the crucible does not demand the undivided attention of the analyst, but is simply left to itself till oxidation is complete, it is possible to carry out simultaneously any

number of combustions. Almost any of the ordinary Kjeldahl digesting shelves can be adapted to carry a series of crucibles. One has then but to light the burners under them one after another, and return to extinguish them in the same order. There are none of the disagreeable features, attending fusion with sodium hydrate. No annoying rush of inflammable gases escapes through the upper layer of carbonate, and the odor of burning is relatively so slight, that the operation could be carried out in the open laboratory without serious inconvenience.

The rapid mode of fusion here recommended is not only convenient; it is also, what is of course of far greater importance, absolutely safe. It is not attended, so far as I have ever been able to detect, by any risk whatever of a loss of iodine; not even when the temperature has been raised to a bright glowing red. Nothing consequently would be gained by a more gradual incineration. On the contrary, in controls where the heating was spread, by very gradual increase of the temperature, over an hour or an hour and a half, I have not infrequently obtained results considerably too low. Oswald[1] also appears to be of opinion, that a *rapid* oxidation is least likely to lead to loss of iodine. Of course what is here said would not apply to iodine in every form of combination; I am convinced of its truth in the special case under consideration.

As soon as the contents of the crucible are sufficiently cool they are dissolved in water. It has already been stated that complete destruction of organic matter is attained before there is more than a commencement of actual fusion. The mass is therefore generally in large part friable and much more easily brought into solution than the hard, solid cake produced by fusing with caustic alkali. Solution is aided if necessary by the application of heat. Filtration can be entirely dispensed with; there should not be more than a few grains of unburned carbon present, and these do not interfere with subsequent operations. This is another point where a saving of time is effected. On the Baumann plan filtration is an unavoidable preliminary to the employment of colorimetry; and the filtration of a strongly alkaline fluid is a time-consuming operation.

The solution is transferred, then, without filtration, but with every quantitative precaution, to an Erlenmeyer or ordinary flask of 500 cc. capacity. It should be perfectly colorless. Whether or not it is likely to contain any considerable proportion of iodate, is a question, which has not

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[1] Oswald: *Zeitschr. f. physiol. Chem.*, xxvii, p. 31.

been investigated.[1] Since *all* the iodine is to be subsequently raised to that state of oxidation, the point possesses obviously no importance.

SECOND STAGE—OXIDATION OF THE IODINE AND ITS DETERMINATION AS IODIC ACID.

In Baumann's method the iodine, present in the fusion product as sodium iodide, is liberated by acidification, taken up in chloroform or other suitable solvent, and estimated colorimetrically on the principle first proposed by Rabourdin.[2] The accuracy of the determination depends of course, in the first place, on the completeness with which the iodine has been combined in iodide form,—a point which, as we have seen, is open at least to discussion. Apart from this the process presents in itself some inconveniences. It calls for apparatus, in the shape of carefully matched glass cylinders, which may not always happen to be at hand. Colorimetric methods are more liable than almost any others to errors arising from the personal equation. To detect slight differences in the tint of an iodine solution is not a very easy matter; to make it possible at all, one has to first so adjust the amount of solvent used in each analysis that the depth of color lies between certain limits: and even then much practice is required before one attains any degree of confidence in the readings. There are possibilities of error too, as it appears to me, in the circumstances that the extracted fluid cannot, in the nature of the case, have always just the same composition, and that however much the conditions under which the standards are prepared may approximate to those of an actual analysis, they cannot be made identical.[3]

At the same time it is admitted that the colorimetric method has much to commend it and that in the hands of those who have acquired familiarity with its technique, it gives not unsatis-

[1] There is little doubt that it very often does. Harnack (*Zeitschr. f. physiol. Chem.*, xxiv, p. 418) found, in a similar case, that of iodospongin, that fusion with soda and saltpeter yielded always less than the required amount of silver iodide; and Winternitz (*Zeitschr. f. physiol. Chem.*, xxiv, p. 430) observed constant iodate formation, when iodine-containing urine residues were incinerated with sodium carbonate.

[2] Rabourdin; *Annalen d. Chem.*, lxxvi, p. 375.

[3] Cf. footnote 3 on page 323.

factory results. There is nothing to prevent its being combined, if desired, with the mode of fusion just recommended. It would probably be necessary in such a combination to first eliminate the possibility of errors due to iodate formation; this could doubtless be effected by applying to the solution, before acidification, the reduction process of Riggs.[1]

For my own purpose I found it more convenient to abandon colorimetry altogether, and attempt the working out of an alternative procedure. The minuteness of the quantities involved ruled out from the beginning all gravimetric methods; the alternative had to be therefore a titrimetric one. There are few who would not, other things being equal, prefer a volumetric scheme of analysis to a colorimetric; and as the plan adopted can claim some superiority of accuracy over the older one, and is for large series of analyses at least as rapid, I believe it may prove in many quarters a not unwelcome substitute.

It is at once obvious, that direct titration of the iodine liberated on acidification is not permissible. There are present too many other substances capable of reacting with thiosulphate. It would perhaps be possible to adapt to the problem the well-known method of Fresenius,[2] wherein the iodine, liberated through the action of nitrous acid, is taken up in carbon disulphide, and titrated with a specially standardized thiosulphate solution. This plan was made use of by Oswald[3] to determine the iodine content of thyreoglobulin, and appears to have yielded him excellent results. For the measurement of the considerably (ten or more times) smaller amounts likely to be met with in thyroid substance, it does not seem to be so suitable. At any rate my own experience with it is unfavorable to its usefulness when the iodine present is less than half a milligram. The very small volume of thiosulphate required in such a case introduces of itself considerable possibilities of error. (One cc. of $\frac{N}{200}$ thiosulphate = 0.63 mgm. iodine). The technique moreover, involving, as it does, repeated extraction and wa hing of the extracts followed by the troublesome titration in a stoppered

[1] Riggs: *loc. cit.*

[2] Fresenius (trans. Cohn.): *Quantitative Chemical Analysis*, New York, 1906, i, p. 537.

[3] Oswald: *Zeitschr. f. physiol. Chem.*, xxxii, p. 124.

bottle, renders the performance of a large series of analyses a very laborious and time-consuming undertaking. Finally, the method would fail completely if much iodate formation had occurred.

There exists now another, less frequently employed procedure for the estimation of very small amounts of soluble iodide. It is generally associated with the name of Dupré[1] and is based upon the fact that if to a solution containing iodine there be cautiously added chlorine water, the iodine at first set free is by further addition of the reagent converted quantitatively into iodic acid.

$$I_2 + 6H_2O + 5Cl_2 = 10HCl + 2HIO_3$$

The point at which the iodine is completely converted into iodic acid can be determined quite sharply by the disappearance of the starch or chloroform reaction. The amount of a standardised chlorine water consumed in reaching this end-point gives accurately the amount of iodide present.

It is clear that this method, in the form described, is not applicable to cases where the iodine exists already free or as iodic acid, or where any organic or inorganic substance capable of reacting with chlorine is at hand. All these conditions are, or may be, present in the case we are concerned with. Dupré, in dealing with mother liquors containing traces of organic matter, proposed to get over such a difficulty in the following way; he added of any weak chlorine solution *just enough* to lead to the disappearance of the iodine reaction; the iodine was thus converted as before into iodic acid, while simultaneously all interfering substances were oxidized. On adding now an excess of potassium iodide, six atoms of iodine were immediately liberated for every one originally present.

$$5KI + HIO_3 + 5HCl = 5KCl + 3H_2O + 3I_2$$

The free iodine could be titrated with sodium thiosulphate, and with this advantage, that there was six times as much to titrate as the solution had contained to begin with.

[1] Duprè: *Annalen d. Chem.*, xciv., p. 365.

On this second plan it is of course a matter of complete indifference, whether the iodine was at first free, or combined as iodide or as iodate. It appeared to me not unlikely, therefore, that it could be successfully applied to the acidified solution of the carbonate fusion. The results obtained on making a series of trials were, however, very disappointing. The point at which chlorine had been added in just sufficient quantity was found to be difficult of precise determination; and the solution, after the addition of potassium iodide, did not prove to be a suitable medium for the titration. There was no definite end-point, and the results did not even show constancy in the amount of error.

These difficulties were surmounted by the introduction of a simple modification. Instead of attempting to use precisely the required amount of chlorine and no more, chlorine was added at once in excess and the surplus was afterwards removed by boiling. This treatment converted the iodine as before into iodic acid, which is non-volatile, while all interfering substances (nitrous acid, sulphur compounds, etc.) were either oxidized or driven off. Addition of potassium iodide led now to the quantitative liberation of the theoretical amount of iodine, and no difficulty was experienced in titrating it with sharpness.

The admissibility of this alteration of Dupré's original procedure was proved by preliminary tests on pure solutions of potassium iodide. These were treated with excess of chlorine water, boiled for half an hour and, after cooling and the addition of potassium iodide, were titrated with freshly standardized solutions of sodium thiosulphate. Table I exhibits the sort of result obtained.

The suggested procedure gave then quantitative results with simple iodide solutions. It had to be next determined whether it was applicable under the special conditions of the problem in hand. To this end a solution was prepared containing 1.661 grams of pure dry potassium iodide to the liter. From a fine, carefully calibrated, 1 cc. pipette reading to hundredths and permitting the estimation of thousandths, measured amounts of this solution or of its tenfold dilution were added to 1 gram of casein already mixed in the nickel crucible with 16 grams of the oxidation mixture. As soon as the added water had combined with the anhydrous carbonate, the whole was intimately mixed

TABLE I.

MGS. IODINE PRESENT.	CC. THIOSULPHATE.	MGMS. IODINE FOUND.
6.370	64.80*	6.362
6.370	64.95*	6.373
1.270	6.08†	1.263
1.079	5.18†	1.076
1.079	5.21†	1.082
1.016	4.89†	1.015
0.509	2.55†	0.529
0.254	1.26†	0.261
0.169	8.30‡	0.172
0.113	5.51‡	0.114
0.056	2.63‡	0.055

* 1 cc. = 0.589 mgm. iodine; standardized with pure iodine.
† 1 cc. = 1.246 mgm. iodine; standardized with potassium bichromate.
‡ Tenfold dilution of the last mentioned.

together with a clean nickel spatula. The mixture was combusted, dissolved and acidified, and the resulting fluid was treated exactly as described above. The results obtained were encouraging. They were, however, by no means so good as those with iodide solutions. There was uniformly a deficit amounting sometimes, especially with the larger amounts of iodine, to as much as 10 per cent. Although in dealing with quantities so minute such a deficit is hardly surprising and although Oswald[1] and Wells[2] do not claim for the Baumann method a much greater *average* accuracy, yet I did not feel satisfied that the loss was altogether unavoidable. It appeared to me not unlikely, that it was caused at least in part by the effervescence (greater than with the Baumann fusion) which occurs on acidification. If this were the case, it might be expected that the presence of excess of chlorine at the very moment of liberation of the iodine would tend to prevent the escape of the latter. To attain this condition it would be sufficient to add sodium hypochlorite before acidification, instead of chlorine water after it. (Sodium hypochlorite oxidizes iodides to iodates even in alkaline solution, so that its use is doubly advantageous.)

[1] Oswald: *Zeitschr. f. physiol. Chem.*, xxiii, p. 276.
[2] Wells: *loc. cit.*

Whether the premises were correct or not, the result entirely justified the argument; and with this final improvement the method proved capable of a degree of accuracy that leaves little to be desired.

As before, the usefulness of the procedure was tested first by controls upon simple iodide solutions. Table II reproduces some of the results, and indicates for each determination its absolute and percentage error.

TABLE II.

IODINE PRESENT IN MGMS.	IODINE FOUND IN MGMS.	ABSOLUTE ERROR IN MGMS.	PERCENTAGE ERROR.
1.608	1.604	0.004	−0.25
0.884	0.890	0.006	+0.6
0.426	0.429	0.003	+0.6
0.167	0.168	0.001	+0.6
0.089	0.089		
0.044	0.045	0.001	+2.0
0.022	0.026	0.004	+16
0.017	0.017		
0.009	0.010	0.001	+10
0.004	0.004		
0.000	0.000		

The table shows that quantities of iodine at least as low as 0.05 mgm. may, in pure solutions, be determined with almost quantitative exactness; while in the case of even minuter amounts a close approximation can be obtained. The absolute error never rises as high as 0.01 mgm. (The thiosulphate used was, for the first three estimations $\frac{N}{200}$, for the others $\frac{N}{2000}$.) We have here, it may therefore be claimed, an excellent method of measuring minute quantities of iodine and one which presents some advantages not only over the original procedure of Dupré, but also over the better known one of Fresenius. It eliminates the need for chlorine water (standardized or not) in the one case, and in the other the inconveniences attending the use of carbon disulphide. As to its accuracy the figures shown speak for themselves. Of course it does not distinguish between free iodine, iodide and iodate; which may, according to circumstances, be an advantage or the reverse.

The improved method was now applied to casein-iodide mixtures. These were prepared and combusted in the manner already described. To the dissolved product was added in each case sodium hypochlorite; thereupon it was acidified (with phosphoric acid); the excess chlorine was removed by boiling; and the iodic acid produced was determined as before. In Table III are recorded the data of one such series of controls.

TABLE III.

MGMS. IODINE PRESENT.	CC. THIOSULPHATE.	MGMS. IODINE FOUND.	ERROR.
			Per Cent.
1.220	12.35	1.213	−0.6
0.675	6.88	0.676	+0.15
0.348	3.59	0.353	+1.4
0.064	6.61	0.065	+1.5
0.031	3.11	0.031	
0.000	0.20	0.002	

The thiosulphate solution used was, for the first three titrations such that 10 cc. = 5.898 mgms. iodine; for the others, it was ten times weaker.

In spite of the fusion with organic material, and in spite of the complex nature of the solution in which it was ultimately brought to titration, the iodine was determined here with almost as much exactness as in pure watery solutions. To such controls there is *nevertheless one possible objection—it would apply* also to nearly all the controls ever made with Baumann's method—namely, that the iodine was already present in inorganic form. It might be, that organically bound iodine would be recovered less completely. To decide the point, similar tests were made with the addition to 1 gram of casein of minute quantities of an organic iodine compound in place of potassium iodide. The substance with which they were conducted was the sodium salt of diiodoparaphenolsulphonic acid ($NaC_6H_2I_2OHSO_3 + 2H_2O$), commercially known as sozoiodole-sodium. This contains iodine, in what is probably to some extent the same sort of combination as occurs in protein, and resembles protein further in the possession of sulphur in its molecule. The theoretical iodine content is 52.66 per cent. The sample in my possession was, as

gravimetric analysis showed, not quite pure. It was analyzed according to the method of Pringsheim.[1]

0.1830 gram yielded 0.1750 gram AgI,
whence I = 51.68 per cent.

Weighed quantities of this substance were subjected (in the first instance without admixture) to the process under discussion. Ten grams of the carbonate-nitrate mixture were employed. the iodine was liberated at the end by addition of 3 grams of potassium iodide, and the thiosulphate solution (standardized against pure iodine) was of such strength that 10 cc. = 0.1182 grams of iodine.

(1) 0.1572 gram substance required 41.46 cc. thiosulphate whence I = 51.92 per cent.
(2) 0.3265 gram substance required 85.92 cc. thiosulphate, whence I = 51.86 per cent.

The figures show a gratifying agreement, and the mean value (51.89 per cent) differs by only 0.4 per cent from the result of the gravimetric analysis.[2]

Of this same "sozoiodole" two solutions were prepared, which contained respectively 1.351 and 1.387 grams of iodine in the liter. (The gravimetric determination was made the basis of the calculation.) Fractions of a cc. of these solutions, or of tenfold dilutions of the same, were fused along with 1 gram of casein. The steps of the subsequent analysis were those described for the similar experiments with potassium iodide solution and casein. The following table contains the results of all the controls, that were carried out upon this plan.

These figures are, I think, sufficient to demonstrate, that even when iodine is in organic combination, it can be determined with very nearly the same degree of exactness, as if it were originally present as iodide.

[1] Pringsheim: *Ber. d. deutsch. chem. Ges.*, xxxvi, p. 4244; xxxviii, p. 2459.

[2] This result suggests the possibility of applying the principle of the method to the determination of iodine in the elementary analysis of organic substances. The idea is the subject of experiments at present in progress in this laboratory.

TABLE IV.

IODINE PRESENT MGMS.	CC. THIOSULPHATE.	IODINE FOUND MGMS.	ERROR IN THOUSANDTHS OF A MGM.	PERCENTAGE ERROR.
1.301	13.18*	1.298	3	−0.23
1.254	9.90‡	1.240	14	−1.1
0.854	8.42*	0.830	24	−2.8
0.691	5.43‡	0.680	11	−1.6
0.415	4.31*	0.425	10	+2.4
0.128	9.62§	0.121	7	−5.5
0.126	13.3†	0.131	5	+4.0
0.0715	7.0†	0.0690	2.5	−3.5
0.0447	3.26§	0.0408	3.9	−8.7
0.0179	1.46§	0.0183	0.4	+2.2
0.000	0.15§	0.0019	1.9	
0.000	0.00	0.000		

* 10 cc. = 0.985 mgm. "original" iodine. † One tenth as strong.
‡ 10 cc. = 1.253 mgm. "original" iodine. §One tenth as strong.
The thiosulphate solutions were standardized against potassium biniodate.

In order thoroughly to establish the validity of the method, it remains yet to show, that it will yield concordant results in duplicate analyses of thyroid glands. The following examples of such analyses prove it to be capable of doing so. (See Table V.)

Of the analyses here reproduced, numbers 1 and 7 were made on specimens of thyroid tablet, stated to contain 40 per cent of thyroid tissue; numbers 8, 10, 11 and 12 on commercial samples of desiccated thyroid; and the remainder on the glands of sheep, which were the subject of experiment in this laboratory. The twelve form a fair selection from the series of duplicates accumulated during the past year. The table includes the pair showing the poorest agreement yet met with (number 5), and also the pair showing the best (number 11). It includes three somewhat extended sets of parallel determinations (1, 10 and 12), which constitute a rather severer test of the method, than a mere duplicate affords. Taken altogether the results are very satisfactory; with the exception of number 5 they show very much the extent of agreement that the controls of Tables III and IV might have led one to anticipate. In the last column is shown in each instance the greatest percentage deviation from the average

TABLE V.
Analyses of thyroid substance.

NO.	GMS. TAKEN.	IODINE FOUND MGMS.	IODINE PER GM. MGMS.	AVERAGE.	ERROR.
					Per Cent.
1	1.095	0.676	0.617		
	0.900	0.546	0.607		
	1.000	0.603	0.603		
	0.848	0.515	0.606	0.612	2.0
	1.000	0.615	0.615		
	1.023	0.617	0.603		
	1.000	0.624	0.624		
	0.897	0.559	0.620		
2	1.000	2.669	2.669	2.678	0.35
	1.000	2.687	2.687		
3	0.86	3.902	4.537	4.515	0.6
	1.000	4.492	4.492		
4	1.000	1.300	1.300	1.303	0.23
	0.900	1.175	1.306		
5	1.000	0.912	0.912	0.936	2.5
	1.000	0.959	0.959		
6	1.000	1.618	1.618	1.609	0.56
	1.000	1.600	1.600		
7	0.984	0.584	0.594	0.596	0.35
	0.992	0.593	0.598		
8	1.089	2.767	2.516	2.510	0.3
	0.954	2.411	2.503		
9	0.979	2.356	2.407	2.413	0.25
	0.938	2.269	2.419		
10	0.978	1.517	1.551		
	0.998	1.544	1.547		
	1.031	1.585	1.537	1.552	0.97
	1.194	1.861	1.560		
	0.906	1.419	1.567		
11	0.953	1.751	1.836	1.837	0.06
	0.871	1.599	1.838		
12	1.057	1.476	1.396		
	1.048	1.454	1.387		
	0.558	0.794	1.420	1.407	1.4
	0.508	0.723	1.423		

shown by any single determination of the group. Setting aside the exceptional case of number 5, it will be seen, that, whenever the quantity of iodine actually measured exceeds 1 mgm., the error in this sense is always less than 1 per cent; and that the absolute range of the values found for iodine per gram of thyroid is seldom greater than 0.03 mgm. (two exceptions), and usually less than 0.02 mgm. (seven cases).

To two of the analyses there attaches a special interest. Dr. Atherton Seidell of the Hygienic Laboratory, U. S. Public Health and Marine Hospital Service, was kind enough to send me, at my expressed desire, two samples of thyroid powder, of which the iodine content had already been determined by the Baumann process. A direct and unprejudiced comparison of results obtained independently by the two methods was thus made possible. (Dr. Seidell's figures were of course withheld till my analyses had been completed.) The samples supplied to me are numbered 11 and 12 in the table. The iodine per gram found by the Baumann method was respectively 1.80 and 1.07 mgm. In the case of No. 11 the agreement with my own figure will be seen to be very close indeed; and what difference there is, is in the direction that might have been expected. In the other case there is between the two results a divergence which is certainly considerable. Only an elaborate gravimetric analysis would decide positively, which is right; but, as the oxidation method has not in any series of controls shown a tendency to give values appreciably *too high*, I am inclined to look on the larger figure as more likely to be the correct one, and to interpret the results of the comparison as being distinctly in favor of the new procedure.

The principle of the method having been fully justified by the groups of results submitted, it is perhaps not superfluous to describe now, somewhat more minutely than has yet been done, the successive steps of the estimation.

DETAILED DESCRIPTION OF THE IODINE ESTIMATION.

The reagents required are:

(1) Solution of sodium hypochlorite, of which the chlorine value has been roughly determined.

(2) Phosphoric acid solution, made by diluting the 85 per cent syrup with an equal volume of water.

(3) A 1 per cent solution of potassium iodide.
(4) Standard solution of potassium biniodate.
(5) Dilute hydrochloric acid, 1 : 5.
(6) Sodium thiosulphate solution, approximately $\frac{N}{200}$.
(7) Starch solution.

The material under analysis is understood to have been combusted and the product brought into solution, in the manner already described (see p. 324). The bulk of the fluid will be from 150 to 200 cc. If it is less, enough water should be added to bring it within these limits.

To this solution in a 500 cc. flask is added just sufficient sodium hypochlorite to ensure that on acidification there shall be present a moderate excess of chlorine. The correct amount is readily learned by a few trials. It is equivalent to, as a rule, 0.8–1.0 gram chlorine or 30–45 cc. of fresh *Liquor sodae chlorinatae, U. S. P.* By roughly determining the available chlorine of any given sample, the proper quantity to use may be calculated. A large excess is inconvenient, but does no harm. Occasionally the conditions are such as to require greater quantities than those mentioned.

The flask is now held in a slanting position, preferably under the cold water tap, and the contents, to which a continuous rotary movement is communicated, are acidified by the addition of 40 to 60 cc. of the phosphoric acid. Care must of course be taken that the escaping gas-bubbles occasion no loss of liquid; but the acid should not be added *too* gradually. Phosphoric acid is to be preferred to sulphuric; when the latter is used, the results are distinctly less exact. This may possibly be accounted for by the formation, on subsequent boiling, of traces of sulphurous acid.

When effervescence has ceased, the liquid should be faintly yellow, and should smell distinctly though not strongly of chlorine.

A short stemmed funnel having been placed in the mouth of the flask, the contents are vigorously boiled. Bumping, if it should occur, is relieved by the addition of some chips of clean porous porcelain, or a *few grains* of talc. From time to time the escaping steam is tested with starch-iodide paper. The blue reaction should fail in from five to ten minutes; if it disappear sooner, there is likely to have been too little chlorine present; if

it persists longer, there has been certainly more than is necessary. Boiling is continued 15 to 20 minutes longer. Numerous blank controls have shown, that a boiling of this duration is usually necessary and sufficient for the expulsion of the last trace of chlorine.

The volume of fluid has now been reduced to about 150 cc. It is cooled under a stream of cold water, or, if the titration is not to be carried out immediately, set aside till it has reached room temperature. When it is cold, 10 cc. of the 1 per cent potassium iodide solution are added,[1] and thoroughly mixed with the contents of the flask. The liberated iodine is *immediately* titrated with the sodium thiosulphate, freshly standardized in the manner presently to be described. Starch is added, as usual, towards the end of the reaction; if the amount of iodine is excessively small, starch is added from the beginning. The titration is carried to the disappearance of the last visible tinge of blue or pink; this point is most readily observed by looking at the flask fron the side, while it is held upon a white surface with the light falling on it from the front. There should be no reappearance of the blue color for several minutes after the end-point is determined.

The liberation, when iodide is added, of any considerable amount of iodine is of course indicated at once by the yellow color which the liquid assumes. Even with as little iodine (originally present in the material analyzed) as 0.03 mgm., a faint yellowish tinge may be imparted; with 0.05 mgm., the yellow is quite distinct. Smaller quantities than these are rendered evident by the reaction with starch; with only 0.005 mgm., a light blue develops; with 0.01 mgm. a deep blue. These figures have reference to a volume of 150 cc. or thereabout.

In order to attain the most exact results of which the process just described is capable, it is necessary to attend scrupulously to several points of detail. These will now be severally considered.

Purity of reagents. In the first place, it goes without saying that each of the reagents employed must be absolutely free, not only from iodine,[2]

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[1] If a great excess of phosphoric acid has been used, it is best, before adding potassium iodide, to reduce the acidity somewhat by addition of alkali.

[2] Even potassium nitrate may, as I have found, contain minute traces of iodine.

but from anything, which would be capable at the end of the process of liberating iodine from potassium iodide. A blank experiment, run with 1 gram of casein or fibrin or other iodine-free protein material, will settle the question; if on the addition of starch and potassium iodide, more than a just perceptible shade of blue appear (say, more than can be discharged by 0.25 cc. of $\frac{N}{1000}$ sodium thiosulphate), the same test must then be applied to each of the reagents in detail, until the one responsible is found. The reagent that gives most frequent trouble is the hypochlorite solution. It must be reasonably fresh. If this condition can be fulfilled, the commercial product often gives excellent results. It yields a precipitate of calcium salts, when added to the alkaline solution, but this dissolves again on acidification. Sometimes, however, it is impossible to get a commercial solution, that does not give values slightly too high. There is then nothing for it, but either to apply a correction for each analysis, or, better, though more troublesome, to prepare one's own hypochlorite in the laboratory. This is done by passing chlorine into a 10 per cent solution (well cooled) of pure sodium hydroxide *e natrio metallico.*

Controls. Even if the purity of the reagents is assured, it is well to accompany each set of actual determinations by a blank control with casein. Obviously one must never be in any doubt, as to whether the excess of chlorine used has been thoroughly expelled. While the time prescribed for boiling (about half an hour in all) has in my experience always proved sufficient, and no one of the many controls made has ever given more than a minimal *plus* of iodine (the error being usually in the opposite direction), yet certainty upon the point can be felt only when one carries out a blank experiment under similar conditions. It has to be noted, that only the *immediate* result of adding the potassium iodide and starch is of importance here. All controls of this sort develop a blue color sooner or later on standing. Many, moreover, do show at once a perceptible tinge; the maximum significance of this on titration is shown in the last figure of Table III, and it will be seen that for all usual amounts of iodine it is quite unimportant.

The starch solution. As the thiosulphate solutions used are very dilute, it is important to have an indicator as sensitive as possible. Different samples of starch differ considerably in their behavior towards very minute traces of iodine. I am indebted to Professor Orndorff of this University for pointing out to me the excellent qualities in this respect of pure arrow-root starch. A 0.5 per cent solution, of which 5 cc. may be used, furnishes an indicator, that is not surpassed in delicacy by any other variety of starch, which I have tried. It is convenient to prepare a considerable quantity of it at once, allow it to stand over-night in cylindrical vessels and decant into test tubes or small bottles; these are then sterilized by heating for an hour in boiling water, and sealed by plugs of sterile cotton wool. So prepared, it will keep indefinitely, until the plugs are removed.

The thiosulphate solution. This must necessarily be very dilute. In early experiments with the method, I attempted to prepare it of such

strength, that 10 cc. were equivalent to 6 mgm. of iodine, i. e., to 1 mgm. of "original" iodine. Such a solution would contain 1.1735 grams in 1 liter, and would be very nearly $\frac{N}{200}$. The burette reading would give directly the quantity of iodine, and no calculation would be required. Unfortunately for the scheme very dilute thiosulphate solutions rapidly suffer decomposition, and do not retain the same value for more than a few days together. It is therefore pure waste of time to make one of any given titer to start with.

The most convenient way of proceeding is to make up a stock solution containing about 25 grams to the liter. By diluting this roughly twenty times one obtains a thiosulphate of suitable strength for the actual titration in the majority of cases. This is to be standardized afresh at least every second day on which it is made use of.

The standardization does not involve so great a complication of the method as might be imagined. The ultimate standard must of course be a given weight of pure iodine. This cannot be applied in the form of a stock solution of known concentration, for dilute iodine solutions are as little permanent as dilute thiosulphate. On the other hand the method would be rendered altogether toc tedious, if standardization required the actual weighing out, before each series of titrations, of a definite amount of iodine. Fortunately this is not necessary. The addition of potassium biniodate to an acidified solution of potassium iodide leads to the liberation of six times as much iodine as its own molecule contains.

$$KIO_3.HIO_3 + 10KI + 11HCl = 11KCl + 6H_2O + 6I_2$$

As this reaction takes place instantaneously and quantitatively, we have in a properly standardized biniodate solution the means of procuring at *any moment an accurately known amount of pure free iodine.*[1]

A biniodate solution of suitable strength for the present purpose is obtained by dissolving the salt in the ratio of 0.2 to 0.3 grams to the liter. This, though very dilute, is absolutely permanent. Its exact value having been once determined, it serves as the standard for all subsequent measurements. It is well therefore to prepare several liters of it at once.

Standardization of the biniodate. If one is certain of the complete purity of the potassium biniodate, the solution can be prepared by dissolving an accurately weighed amount in a known volume of water. It is safer to adopt iodine as the ultimate standard, and compare the biniodate with that. This may be done in several ways, of which the following is one. A solution is prepared, containing about 0.5 gram (accurately weighed) pure, dry, freshly sublimed iodine, and 2 grams of potassium iodide in one

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[1] See v. Than: *Zeitschr. f. anal. Chem.*, xvi, p. 477. Potassium bichromate (Zulkowski: *Journ. prakt. Chem.*, ciii, p. 362), or potassium permanganate (Volhard: *Annalen d. Chem.*, cclxii, p. 98) can also be employed to liberate iodine in definite amount; but the biniodate has proved more satisfactory than the former, while with the latter I have no experience.

liter of water.[1] A measured volume of this is pipetted into 150 cc. of water in an Erlenmeyer flask; a little dilute phosphoric acid is added, and the iodine is titrated with an approximately $\frac{N}{200}$ thiosulphate solution; starch is used as indicator, and the thiosulphate is added to the complete disappearance of any trace of blue or pink.[2] By a series of such comparisons the titer of the thiosulphate is accurately ascertained. Into another Erlenmeyer are now placed 10 cc. of 1 per cent potassium iodide, and 5 cc. of dilute hydrochloric acid (1:5). Twenty-five cc. (or other convenient volume) of the biniodate are run in from a pipette. The contents of the flask are mixed and diluted to 150. cc. The quantity of iodine that has been liberated is then determined by titration with the thiosulphate, whose value has just been learnt.

Standardization of the thiosulphate. Any thiosulphate subsequently made use of is to be standardized against the (now known) amount of iodine liberated, in precisely the same way, and under precisely the same conditions, by a measured volume of the biniodate. The operation is identical with that just described.[3] It can be completed in duplicate

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[1] It may not be superfluous to indicate one way in which this may be done. A liter flask is filled to the mark with water. Into this is carefully dropped a narrow weighing tube containing 2 grams of potassium iodide, and 0.5 cc. of water; with a glass rod the tube is gently pushed under the surface of the water, the stopper is slid in after it, and the new level of the water is marked on the neck of the flask. The weighing tube is now removed and dried; 2 grams potassium iodide and 0.5 cc. water are again put into it; and it is stoppered and weighed. About half a gram of pure, dry, freshly sublimed iodine is introduced, and it is weighed again. The difference gives the amount of iodine. The tube is now held in the neck of the liter flask, into which has been placed about 200 cc. of water. The stopper is loosened, and the tube is allowed to slide down into the water, with the stopper immediately following. (See Treadwell, tr. Hall: *Analytical Chemistry*, 2d. English edition, vol. ii, p. 508.) By gentle agitation the iodine solution in the tube is distributed throughout the water, and the flask is filled to the mark already made. The volume of the iodine is neglected.

[2] In this, as in all the standardizing operations described, the endeavor is made to have all the conditions (volume of fluid, acidity, concentration of iodine), approximate as closely as possible to those under which the actual analyses will afterwards be carried out.

[3] An example may illustrate. The biniodate solution, which has served as standard for most of the titrations recorded in this paper, contains, as was determined in the way described, 0.3389 gms. to the liter. The quantity delivered by a certain "25 cc." pipette, whose actual value is 24.91 cc., sets free therefore 33.18 mgms. of iodine. Now of one thiosulphate solution that was used, it was found, that in two experiments 48.05 and 48.01 cc. (average = 48.03 cc.) were required to titrate that

within ten minutes, and its necessity offers therefore no serious drawback to the employment of the method.

If the amount of iodine in the sample analyzed is less than 0.3 mgm. (a circumstance which one can with a little experience recognize from the depth of color imparted to the liquid) it is advisable to use a thiosulphate solution even weaker than $\frac{N}{200}$. This is prepared *ex tempore* by diluting the already standardized stronger one. I have employed in all such cases only one dilution, and that a tenfold one. Obviously intermediate ones could also be employed. It is true that with the $\frac{N}{2000}$ solution the end-point becomes rather indefinite; but the error arising from this fact is considerably smaller than that occasioned by a single drop of the more concentrated thiosulphate.

It is clearly always of advantage, to so arrange matters that a large number of titrations fall to be done at the same time. They can then be carried through more rapidly than an equal number of colorimetric determinations.

LIMITS OF THE METHOD IN REGARD TO ACCURACY AND DELICACY.

In dealing with such excessively minute amounts of iodine as are actually met with in most samples of thyroid gland (amounts of the order of 0.1 per cent), it is almost too much to hope, that a method could be devised, which should be rigidly quantitative in its results. The utmost that can be reasonably required of a new procedure, is that it shall be at least as exact as previously existing ones. Something more than this can, I believe, be claimed for the method here advocated.

It is difficult to ascertain from the literature just what degree of accuracy is claimed for the Baumann process. The data on which a judgment might be formed are somewhat meagre. One would wish to know at least (1) what results are obtained on material containing a known amount of added iodine, (2) what measure of agreement is met with when parallel determinations are made on a single sample of iodine-containing tissue, (3) what is the smallest absolute amount, and what is the smallest difference, of iodine, that the method enables us to measure.

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quantity. The iodine equivalent of the thiosulphate was accordingly determined as, 1 cc. $= \frac{33.18}{48.03}$ mgms. $= 0.6908$ mgms.; or 1 cc. represented 0.115 mgm. of iodine in the substance analyzed.

(1) As to the first point, Baumann[1] records only two controls (with potassium iodide and fibrin), in which he recovered respectively 84 and 90 per cent of the iodine. Oswald[2] in four similar tests with amounts varying *from 0.1 to 1.0 mgm.* (potassium iodide) obtained with improved technique 80 to 98 per cent. Riggs[3] analyzed about thirty mixtures of potassium iodide and fibrin, with a range of from 0.45 to 4.0 mgms. of iodine, and found that in the majority of cases 90 per cent or more of the iodine could be recovered by the Baumann method.

(2) In regard to the second point, it is curious that there exists, so far as I have been able to discover, no published record of a reasonably extensive series of parallel determinations upon one sample of thyroid. Riggs exhibits results tending to show that in single determinations from 2 to 77 per cent (!) of thyroid iodine may escape the unmodified Baumann technique altogether; and he gives one duplicate pair. where the disagreement amounted to 30 per cent of 2 mgms.[4]

(3) The smallest difference that Baumann himself claimed to be susceptible of measurement was 0.076 mgm. of iodine, Improvements introduced since his time have undoubtedly lowered this limit. Wells,[5] e. g., states that a difference of 0.02 mgm. can be readily observed and he gives that amount as the smallest that can be detected in 1 gram of substance.

Dr. Atherton Seidell, whose experience with the Baumann method has been very extensive, has been good enough to furnish me privately with further information on some of the points just mentioned. He states, that it is possible, by reducing to 1 cc. the amount of the extracting agent employed, to detect as little as 0.01 mgm. of iodine per gram; possibly, by taking special precautions, 0.005 mgm.; and that in duplicate determinations affecting larger quantities of iodine, a variation of 0.05 mgm. is about what might be expected. "Much greater differences may easily arise unless care is exercised."

[1] Baumann and Roos: *loc. cit.*
[2] Oswald: *Zeitschr. f. physiol. Chem.*, xxiii, p. 276.
[3] Riggs: *loc. cit.*
[4] Cf. however, Seidell, *loc. cit.*
[5] Wells: *Zeitschr. f. physiol. Chem.*, xlv, p. 413.

On the ground of the statements collected above, I believe one is justified in concluding for the Baumann method; (1) That it is capable of detecting 0.01 mgm. of iodine per gram of thyroid and (2), that the error involved in its use is with 1 mg. of iodine about 2 per cent of the whole amount, and with only 0.1 mgm. increases to possibly 20 per cent. With still smaller quantities the error would probably be even larger.

If we consider now the principle and technique of the alternative method that has been described, it becomes evident, that its accuracy and delicacy are conditioned by the following factors:

(1) The degree to which the risk of losing iodine by volatilization, etc., is eliminated at the several stages.

(2) The certainty with which we can exclude the presence, in the final operation, of substances (other than iodic acid), which would liberate iodine from potassium iodide.

(3) The limit to the sensitiveness of the iodo-starch reaction.

(1) That the liability to actual loss of iodine is extremely slight is evident at once from an inspection of the control results of Tables III and IV. The greatest deficit there recorded amounts to 0.024 mgm. (out of 0.85). In not one of the numerous other controls, which I have made, has the error in this direction risen to 0.03 mgm. It may be taken as proved then, that none of the operations of the method is attended by any serious risk of loss.

One possibility does, nevertheless, seem worthy of separate consideration. The use of sodium hypochlorite solution introduces also considerable quantities of chloride. In the subsequent operations a certain amount of hydrochloric acid is bound therefore to be liberated. Now iodic acid is decomposed by hydrochloric acid in the manner indicated in the equation,

$$HIO_3 + 5HCl = 2Cl_2 + ICl + 3H_2O.$$

The reaction is, however, one that requires a large excess of hydrochloric acid to be present, before it will take place. It is practically impossible in the case we are concerned with that the necessary concentration should ever be produced. The point was subjected directly to a series of tests. Identical quantities

of iodic acid were added to

1. 150 cc. water
2. " "
3. " " + 3 cc.. conc. HCl
4. " " + " " " + 4 gms. KNO_3
5. " " + 2 gms. NaCl + 4 gms. KNO_3 + 5 cc. H_3PO_4
6. " " + 6 gms. " + " " + " "
7. " " + 10 cc. conc. HCl.

All but 1 were boiled for 20 minutes, and all were then treated with potassium iodide, acidified if necessary, and titrated with $\frac{N}{100}$ sodium thiosulphate. The quantity used was

1.	16.18 cc.	2.	16.20 cc.
3.	16.08 "	4.	16.00 "
5.	16.13 "	6.	16.07 "
7.	7.30 "		

Number 3 contained the utmost amount of hydrochloric acid, that would ordinarily be introduced, and it will be seen, that even as free acid it accomplishes little destruction of iodic acid. In 5 the actual conditions of the method are most nearly imitated, and no measurable loss of iodine has taken place. These observations were repeated with practically identical results. Finally, to clinch the matter, the same quantity of iodic acid was added to the solution obtained on fusing 1 gram of casein in the regular way; after treatment with sodium hypochlorite and phosphoric acid, etc., the titration demanded 16.25 cc.

(2) The second point has already been to some extent discussed in speaking of blank controls. (See p. 339.) It was then stated, that the traces of chlorine, that may sometimes remain after the prescribed time of boiling, are too small to affect the estimation of ordinary amounts of iodine. They are equivalent in the worst case to 0.002 mgm. of that substance. Only with minimal quantities would this have any significance at all; and since, even then, the value found is rarely too high it is clear that this source of error, when it exists, does not do more than cover the unavoidable loss.

To controls performed with pure casein, it might be objected that they fail to take into account the possible influence of one element, namely iron, which is certain to be present to some extent in every actual analysis of tissues, which is not removed

at any stage of the process as described, and which is able under certain conditions to liberate iodine from potassium iodide. The iron could probably be entirely removed by filtering efficiently the alkaline solution of the fusion mass. To do so is not really necessary. The reaction between ferric iron and potassium iodide, represented by the equation,

$$FeCl_3 + KI = FeCl_2 + KCl + I,$$

is an incomplete and reversible one. The point at which it comes to a standstill, and the rate at which this point is reached depend on a variety of conditions, such as the concentration of the reacting substances, and the temperature and acidity of the mixture. If, e. g., the dilution be very great, the proportion of iodine actually set free will be correspondingly small, and the rate of its liberation very slow. Seubert and Dorrer[1] found that with a concentration (of iron) of 1 in 25,000, the free iodine amounted after 48 hours to only 1.25 per cent of the theoretical yield. Carl Mohr too states that 1 part of ferric chloride in 12,289 of water gives with starch and potassium iodide no color whatever till after the lapse of a considerable period of time.[2] Now, from 1 gram of dried thyroid one is not likely to obtain more than 0.1 mgm. of iron. This will be distributed throughout at least 100 cc. of water; making the concentration only 1 in 1,000,000. No appreciable effect upon potassium iodide could be expected from such extreme dilution.

Even if the quantity of iron to be expected were very much greater than it is, it would for another reason altogether be unimportant. As a matter of fact one can *add* to a casein control considerable amounts of ferric chloride, without being able to detect a trace of liberated iodine. This is accounted for by the circumstance that the acidity is produced by phosphoric acid. If 10 cc. of 1 per cent potassium iodide be diluted with 150 cc. of water, acidified with hydrochloric acid, and treated with starch and a drop of 3 per cent ferric chloride, an almost immediate blue color will develop; while, if the experiment be repeated with phosphoric acid in place of hydrochloric, the liquid will

[1] Seubert and Dorrer: *Zeitschr. f. anorg. Chem.*, v, p. 421.

[2] Carl Mohr: *Annalen d. Chem.*, cv, p. 56.

remain absolutely colorless in the presence of 10 cc. or more of ferric chloride.

(3) If it can be taken as demonstrated, that the method has inherent in itself no really important source of error, its accuracy will be dependent finally upon the degree of exactness attainable in the titration. Now the sensitiveness of the iodo-starch reaction is so great, that in using a thiosulphate of which 10 cc. = 6 mgm. iodine (the strength appropriate for all amounts above 0.3 mgm. "original" iodine) one should have no difficulty in determining the end-point within a single drop (= 0.05 cc.).[1] The error involved in the burette reading will therefore be less than this, say ± 0.03 cc. In a total reading of 10 cc. (= 1 mgm. "original" iodine) this would be 0.3 per cent; in one of 3 cc. (= 0.3 mgm.) it would be 1 per cent. With a ten times diluter solution, as used for amounts below 0.3 mgm., the end-point is less definite. It can generally be fixed within four drops. That means an uncertainty of ± 0.1 cc., which is equivalent to 0.3 per cent of 0.3 mgm., and 10 per cent of 0.01 mgm. Unless the quantity of iodine is smaller than 0.1 mgm., the unavoidable error of titration should not, therefore, exceed 1 per cent.

There is of course a limit to the amount of iodine which can be detected at all by means of starch. This limit is influenced by several factors, among the most important of which is the concentration of soluble iodide or hydriodic acid present. With the concentration prevailing in the present case (purposely chosen so as to enhance as far as possible the sensitiveness of the starch reaction[2]), the smallest quantity of iodine, that will produce a perceptible color, is, as I have determined by direct addition of iodine to acidified solutions of the fusion mixture, 0.03 mgm. Consequently, if the sample of tissue under analysis contains less than one-sixth of this (= 0.005 mgm.) it would fail to become evident.[3] Here then would be in theory the utmost limit of deli-

[1] Neumann (see *Zeitschr. f. physiol. Chem.*, xxxvii, p. 124) got sharp results with $\frac{N}{250}$ thiosulphate.

[2] Compare on this point Andrews: *Journ. Amer. Chem. Soc.*, xxxi, p. 1038.

[3] While 0.03 mgm. is the smallest quantity, that will give any starch reaction, yet once that lower limit has been passed, much smaller *differences* are distinguishable.

cacy for the present method. We have seen, though, that even in the entire absence of iodine, there is sometimes a tinge of color. For safety's sake, therefore, the practical limit must be placed at least twice as high as the theoretical one; and, unless the blue produced on adding potassium iodide is quite decided, and requires at least 1 cc. of the $\frac{N}{2000}$ solution to discharge it, it would be unwise to conclude definitely for the presence of iodine. That is to say, smaller amounts than 0.01 mgm. are not to be reported. With quantities of this order one would in any case repeat, if possible, the analysis with a larger weight of material.

Reviewing the considerations of the foregoing paragraphs, and comparing with them the actual reported results of control and thyroid analyses (Tables III, IV, and V), one may, I think, on the question of the method's accuracy, sum matters up in the following way:

(1) If the amount of "original" iodine measured be 1 mgm. or more, the error of a single determination is not likely to be greater than 1 per cent of the whole.

(2) If it lie between 1 mgm. and 0.1 mgm. the error will probably be less (and is often much less) than 5 per cent.

(3) If it be smaller than 0.1 mgm., the error will not usually exceed 10 per cent.

(4) The method will detect and approximately measure as little as 0.01 mgm.

Of course, if duplicate determinations are made, as they should be whenever the amount of available material permits it, the probabilities of error are still further diminished. With ordinary quantities of iodine (1 to 2 mgms.) duplicates as already stated seldom differ by more than 0.03 mgm. That occasionally the disagreement does exceed this limit, is hardly to be wondered at; but even the maximum divergence I have met with (see nos. 3 and 5 of Table IV) is still but 0.05 mgm.

It is clear from the above, that it would be useless in actual practice to report the values found more closely than to hundredths of a milligram; unless, indeed, the amount falls below 0.2 mgm., when the thousandths begin to have some significance. If in most of the analyses recorded in this paper the decimals have been given at greater length, it is merely with the object of elucidating more exactly the real limitations of the method.

SUMMARY.

(1) In a solution containing traces of iodine, whether free, or as iodide or iodate, it is possible by the use of a somewhat modified Dupré method to determine rapidly, conveniently and accurately the total iodine present.

(2) By combining the procedure with the mode of fusion described, one can estimate the iodine content of thyroid gland or other tissue in a way which possesses over the Baumann method the following advantages:

(*a*) The combustion is carried out in an expeditious, cleanly, and convenient manner, which permits the ready simultaneous performance of a large number of analyses.

(*b*) The whole process, especially when a series of determinations is undertaken, consumes less time, and calls for less continuous attention on the part of the analyst.

(*c*) The possibility (be it remote or otherwise) of error due to iodate formation is entirely eliminated.

(*d*) The colorimetric method of estimating the iodine is replaced by a volumetric one; and as the quantity is sixfold multiplied before it comes to titration, the unavoidable error of the observation is correspondingly diminished.

(*e*) The results, particularly with the smaller amounts of iodine, are a closer approximation to quantitative accuracy.

(In concluding, I desire to acknowledge my indebtedness to Dr. Atherton Seidell, of the Hygienic Laboratory, Washington, for the very friendly way in which he placed at my disposal the information and the material in his possession.)

CONCERNING THE RELATIVE MAGNITUDE OF THE PARTS PLAYED BY THE PROTEINS AND BY THE BI-CARBONATES IN THE MAINTENANCE OF THE NEUTRALITY OF THE BLOOD.

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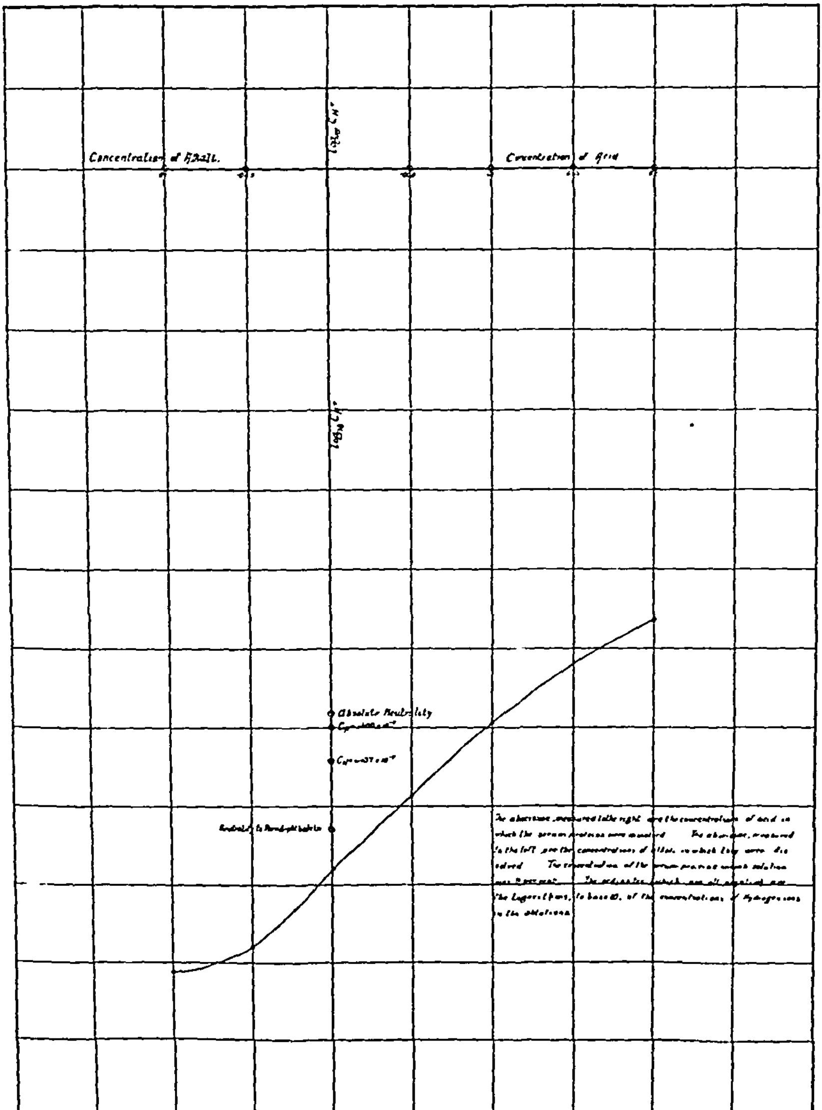
In a recent publication[1], Henderson has directed certain criticisms against the view which has been expressed by me[2] to the effect that the part played by the proteins in maintaining the neutrality of the tissues and of the tissue-fluids, and, in particular, of the blood, is probably comparable with that played by the bicarbonates. Henderson estimates from known quantitative data, the quantity of acid which would be neutralized by the bicarbonates of the blood in passing from the reaction of normal blood ($= 0.37 \times 10^{-7}$ N H^+ at 38°) to that of blood in advanced acid intoxication (about 1.00×10^{-7} N H^+ at 38°). He at the same time, from data derived from experiments in which he employed indicators to determine the change in the reaction of solutions of the serum-proteins to which varying amounts of acid and alkali had been added, estimates the amount of acid which is neutralized by the proteins of the blood as the reaction of their solutions changes by the same amount, and he concludes that it cannot be more than one-fifth of the amount of acid which would be neutralized by the bicarbonates.

It appeared to me, for reasons which will be obvious, that experiments involving the use of indicators to determine such slight changes of H^+ concentration in protein solutions afforded a very

[1] L. J. Henderson: this *Journal*, vii, p. 29, 1909.
[2] T. Brailsford Robertson: this *Journal*, vi, p. 313, 1909.

dissolved, and plot the values of log C_{H^+} given in the above table we obtain the curve shown in the accompanying figure. By interpolating from this curve it is readily seen that in passing from the reaction 0.37×10^{-7} N H^+ to the reaction 1.00×10^{-7} N H^+ 100 cc. of an 8 per cent solution of the serum-proteins neutralizes 2.25 cc. of $\frac{N}{10}$ hydrochloric acid, that is, 22.5 cc. or about one-fifth of its own volume of $\frac{N}{100}$ hydrochloric acid, which is Henderson's maximum estimate. According to Henderson, in passing through the same range of H^+ concentrations the bicarbonates in 100 cc. of blood will neutralize the equivalent of 100 cc. of $\frac{N}{100}$ hydrochloric acid. Hence we must conclude that, between the reactions mentioned, the proteins of the serum are only one-fifth as efficient in maintaining its neutrality as the bicarbonates. To the neutralizing power of the serum-proteins must be added in circulating plasma, that of the fibrinogen; this is, however, probably very slight, since only a very small percentage of fibrinogen is contained in blood.

It would not be safe, however, to conclude from the above results that the part played by the proteins in maintaining the neutrality of the *tissues* or of tissue-fluids other than the blood is not possibly equal in magnitude to or even greater than that played by the bicarbonates and acid phosphates. Thus I have recently found that 100 cc. of a 1.5 per cent solution of potassium caseinate, in passing from the reaction 0.31×10^{-7} N H^+ to the reaction 1.14×10^{-7} N H^+ neutralizes the equivalent of 25 cc. of $\frac{N}{100}$ hydrochloric acid; if the amount of alkali bound by casein were directly proportional to the concentration of the casein, then a three per cent solution of casein (the concentration of casein in cow's milk) would neutralize, in passing from the former to the latter of the above reactions, the equivalent of one-half its volume of $\frac{N}{100}$ hydrochloric acid. The part played by the casein of milk in maintaining its neutrality must therefore be very considerable. In the tissues, not only are the proteins more concentrated than they are in the tissue-fluids, but, owing to the predominance of nucleoproteins, their binding-capacity for bases, and, consequently, their power of maintaining the neutrality of their solutions, is probably higher than that of the majority of the proteins which occur in the tissue-fluids. We are not, at present, in the possession of any data which would render an


Concentration of Acid
Absolute Neutrality

estimate of the relative importance of these factors, for the maintenance of the neutrality of the tissues themselves, in the slightest degree reliable.

One fact, of importance to the general theory of protein solutions, should receive mention here. The proteins of serum are evidently precipitated by alcohol, not in the form of the free proteins, but in the form of their salts—it will be observed on referring to the above table, that their solution in neutral $\frac{N}{100}$ potassium chloride is alkaline. Thus one of the constituents of the mixture of proteins obtained from serum by precipitation with alcohol is paraglobulin (the "insoluble" serum-globulin) which, when not combined with bases or with acids, is insoluble in distilled water. Yet the whole of the powder obtained from serum by precipitation with alcohol is soluble in distilled water. If one bubbles carbon dioxide through the solution so obtained a protein, presumably "insoluble" serum-globulin, is precipitated. It will be recollected, in this connection, that van Slyke and Hart[1] found that casein is precipitated by alcohol, from its solution in a solution of a base, in the form of a salt of the base, and that Spiro and Pemsel[2] found that casein is precipitated by ammonium sulphate, from its solution in sodium hydroxide, in the form of sodium caseinate.

CONCLUSIONS.

(1) The concentrations of hydrogen ions in 8 per cent solutions of the serum-proteins, dissolved in solutions of known acidity or alkalinity, have been determined with the aid of the gas-chain.

(2) It is found that in passing from the reaction 0.37×10^{-7} N H^+ (the reaction of normal blood) to the reaction 1.00×10^{-7} N H^+ (the reaction of blood in advanced acid intoxication) 100 cc. of an 8 per cent solution of the serum-proteins at 34° C. neutralizes the equivalent of 22.5 cc. of $\frac{N}{100}$ hydrochloric acid. Accordingly to L. J. Henderson, the bicarbonates in 100 cc. of blood, in passing through the same range of reactions, neutralize the equivalent or 100 cc. of $\frac{N}{100}$ hydrochloric acid. It is concluded that, be-

[1] Van Slyke and Hart: *American Chemical Journal*, xxxiii, p. 461. 1905.
[2] Spiro and Pemsel: *Zeitschr. f. physiol. Chemie*, xxvi, p. 233, 1898–9.

tween the reactions mentioned, the proteins of the blood are about one-fifth as efficient as the bicarbonates in maintaining its neutrality.

(3) It is pointed out that we cannot infer from these results that the part played by the proteins in maintaining the neutrality of the *tissues* or of tissue-fluids other than the blood is not, possibly, equal in magnitude to or even greater than that played by the bicarbonates and acid phosphates.

(4) The proteins of serum, when precipitated by alcohol, are precipitated, not in the form of the free proteins, but in the form of their salts.

ON THE REFRACTIVE INDICES OF SOLUTIONS OF CERTAIN PROTEINS.

I. OVOMUCOID AND OVOVITELLIN.

By T. BRAILSFORD ROBERTSON.

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(Received for publication, March 15, 1910.)

In a previous communication [1] I have shown that the addition of relatively small amounts of casein to watery solutions of acids and bases very markedly increases their refractive indices; that the increase in the refractive index of the solvent is directly proportional to the concentration of casein which is dissolved in it; and that this increase is moreover, when allowance is made for the alteration of the refractive index of the solvent, independent of temperature between 20° and 40°.

The change in the refractive index of a solvent which is brought about by the addition of a solute is a function of the *size* of the molecules of the solute. The large size of the molecules of the proteins, therefore, far from being an obstacle to the attainment of accurate measurements, is, in so far as this physical property of their solutions is concerned, a very positive advantage.

It appears possible that not only may this physical property of their solutions prove to afford a method generally applicable to the quantitative determination of proteins,[2] but also a method of characterising individual proteins, and of estimating the purity

[1] T. Brailsford Robertson: *Journal of Physical Chemistry*, xiii, p. 469, 1909.

[2] I have elsewhere (*Journal of Industrial and Engineering Chemistry*, no. 10, October, 1909,) shown that such a method can be successfully applied to the determination of the percentage of casein in milk. Cf. also Reiss, *Arch. f. exper. Pathol. und Pharm.*, li, p. 18, 1903; *Beitr. z. chem. Physiol. und Pathol.*, iv, p. 150, 1904.

of their preparations or, at all events, of adding certainty to the identification of any given protein.

From this point of view, an extension to other protein-solutions of the investigations, to which I have referred, on the refractive indices of casein-solutions, would appear to be desirable. Accordingly I have carried out the measurements described below.

(*i*) *Ovomucoid.*

The whites of eggs were beaten up to a froth and allowed to stand in shallow vessels overnight. The subnatant fluid was then poured off, the froth being rejected. This fluid was diluted to five times its volume with distilled water, and to every liter of the diluted fluid was added 130 cc. of approximately $\frac{N}{10}$ acetic acid (made up by diluting 10 cc. of glacial acetic acid to 1750 cc.). This mixture was heated slowly to boiling-point, being rapidly and uniformly stirred meanwhile, and, after being allowed to boil for about 3 to 5 minutes, was put aside in rather shallow vessels for about 12 hours. At the end of this time most of the coagulum had floated to the top and the subnatant fluid was filtered through hardened filter-paper. Filtration was very rapid, and the filtered fluid, when boiled, either with or without further addition of acetic acid, remained perfectly clear. The fluid thus obtained was now slowly evaporated to one-fifth of its volume, the temperature of the fluid never being allowed to rise above 55° C. After allowing this fluid to cool, the protein was precipitated from it by the addition of ten volumes of 99.8 per cent alcohol (Kahlbaum's) and was allowed to settle in tall glass cylinders. The supernatant fluid was then syphoned off and the precipitate was washed in the same volume of alcohol as that employed in the precipitation. This washing was repeated, again employing the same volume of alcohol, and the precipitate was allowed to steep in this alcohol for about 24 hours, in order, if possible, to remove all adherent or combined acetic acid. The alcohol was then syphoned off and the precipitate was washed in the same volume of ether (Kahlbaum's Ueber Natrium distilliert). This washing was repeated. The ether was then syphoned off and the thick suspension of protein in ether thus obtained was rapidly poured into a hardened filter, the filter and the contained suspension of protein in ether being at once transferred to an incubator and the filtration continued over sulphuric acid at 40° (to avoid condensation of atmospheric moisture on the filter. It goes without saying that throughout the processes of washing, settling, etc., possible introduction of atmospheric moisture was avoided by keeping the glass cylinders closed with ground glass stoppers.).

After the completion of filtration, the ether which had filtered off was removed from the incubator and the precipitate was allowed to dry for 24 hours. The protein was then obtained in the form of chalky cakes which were very readily broken up into fine, impalpable powder. This powder

was passed through a fine sieve and kept in a glass-stoppered bottle. It was found not advisable to work with fewer than six dozen eggs at one time as, otherwise, the amount of precipitate which is finally obtained is so small that the danger of excessive caking and partial decomposition, in drying, due to the deposition of traces of moisture upon the filter, is very great.

The protein which is thus obtained has been identified by Mörner[1] as a mucoid, and is termed by him ovomucoid.

About a gram of the ovomucoid thus obtained was dissolved in about 100 cc. of $\frac{N}{2}$ hydrochloric acid and this solution was boiled until 30 cc. of fluid had distilled over. This distillate was then tested for acetic acid.[2] It contained a trace of an acid of the fatty series, sufficient to yield a slight coloration with ferric chloride, but insufficient to yield a precipitate of ferric hydrate on boiling or to yield the ethyl-acetate test.

Five grams of the ovomucoid were dissolved in 100 cc. of distilled water and this solution was diluted to the concentrations desired. The refractive indices of these solutions were then measured, at 18° in a Pulfrich Refractometer, reading the angle of total reflection to within 1′ A sodium flame was employed as the source of light.

In the communication referred to above I showed that for solutions of casein the refractive index is connected with the concentration of casein in the solution by the following formula

$$n - n_1 = a \times c$$

n being the refractive index of the solution of casein, n_1 that of the solvent in which it is dissolved, c the percentage of casein in the solution and a is a constant, i. e., the change in the refractive index of the solvent which is brought about by the addition of 1 gram of casein to 100 cc. From the following table of results it will be seen that the same law holds good for solutions of ovomucoid. The refractive index of distilled water at 18° is taken as 1.3333.

[1] C. Th. Mörner: *Zeitschr. f. physiol. Chemie*, xviii, p. 525, 1894. Cf. Hammarsten, *A Text-book of Physiological Chemistry*, Trans. by Mandel, New York, p. 431, 1904.

[2] Professor H. C. Biddle very kindly performed this test for me.

c = Concentration of ovomucoid, per cent.	n = Refactive index of the solution.	$a = \frac{n - 1.3333}{c}$
5.0	1.3413	0.00160
4.0	1.3397	0.00160
3.0	1.3381	0.00160
2.0	1.3365	0.00160
1.5	1.3357	0.00160
1.0	1.3349	0.00160
0.5	1.3341	0.00160

It will be seen that the value of the ratio $\frac{n - 1.3333}{c}$ is constant, between 5 per cent and 0.5 per cent of ovomucoid, and equal to .00160. In other words, the change in the refractive index of distilled water which is brought about by dissolving ovomucoid in it is directly proportional to the concentration of dissolved ovomucoid, and, for a 1 per cent solution, is equal to 0.00160. For casein the value of this constant is .00152.

(*ii*) *Ovovitellin.*

Ovovitellin was prepared in the following manner.[1] Twenty-five yolks of eggs were carefully washed, without breaking the enveloping membrane, in a stream of water, so as to remove all traces of the whites. To the yolks was then added an equal volume of 10 per cent sodium chloride solution, and the solution thus obtained was extracted from ten to twelve times with twice its volume of ether in separatory funnels, continuing the extractions for several times after the ethereal layers failed to yield a precipitate, due to the presence of lecithin, upon the addition of acetone. The complete extraction occupied some two or three weeks. The watery layer which was finally obtained was then poured into twenty volumes of distilled water, and the precipitate of ovovitellin which was thus obtained was allowed to settle in tall glass cylinders. The supernatant fluid was then syphoned off and the precipitate redissolved in 10 per cent sodium chloride and reprecipitated in the same manner. This process was repeated. Finally, the vitellin was dissolved in very dilute sodium hydrate and the solution was filtered, the filtrate being allowed to drop directly into dilute acetic acid, thus reprecipitating the vitellin. This precipitate was suspended in distilled water and allowed to settle in tall glass cylinders; the supernatant water was then drawn off and the washing with water repeated several times; the precipitate was then washed in 6 liters of 99.8

[1] Cf. Osborne and Campbell: *Journ. Amer. Chem. Soc.*, xxii, p. 413, 1900. H. Aders Plimmer: *Journ. Chem. Soc.*, London, Aug. 1908, p. 1500.

per cent alcohol (Kahlbaum's). After allowing the precipitate to settle the supernatant alcohol was syphoned off and the washing in alcohol repeated twice. The vitellin was then washed twice in ether (Kahlbaum's über Natrium distilliert), employing 6 liters each time. The thick suspension of vitellin in ether finally obtained was quickly poured into a hardened filter and allowed to filter and dry over sulphuric acid at 40° for from 24 to 48 hours. The vitellin is thus obtained as a white, somewhat coarse powder.

About a gram of this ovovitellin was placed in about 30 cc. of alcohol and boiled for some five minutes. About 5 cc. of the alcohol, after filtration, was then tested directly for lecithin by the addition of several volumes of acetone. The remainder of the alcohol was evaporated down to dryness and the residue (barely visible) taken up in about 10 cc. of ether. This ether was then tested by the addition of several volumes of acetone. Both tests proved entirely negative, not the slightest opalescence being produced by the acetone; hence the ovovitellin did not contain any lecithin.

The preparation of solutions of ovovitellin is fraught with difficulties. A five per cent solution in alkali ($\frac{N}{10}$ KOH) is a thick jelly, opalescent owing to the presence of a multitude of air-bubbles entangled in it while stirring. In endeavoring to prepare more dilute solutions it is found very difficult to avoid the formation of small lumps of jelly within the solutions, and these are exceedingly difficult to break up and only dissolve with extreme slowness. Although vitellin is soluble in dilute solutions of the strong acids, yet when the powder is directly mixed with an acid solution it will not dissolve, or only does so with extreme slowness.[1] Finally, it was found possible to obtain clear homogeneous 1 per cent solutions of ovovitellin (not more concentrated) by dropping the vitellin from above into the solvent while undergoing violent stirring, and maintaining the stirring for about an hour. Two such solutions were prepared, the one of 1 per cent ovovitellin in $\frac{N}{20}$ potassium hydroxide, and the other of 1 per cent ovovitellin in $\frac{N}{50}$ potassium hydroxide. The first solution had a refractive index (at 18°) of 1.3351, the second had

[1] Exactly the same phenomenon is encountered with casein. Cf. van Slyke and van Slyke: *Amer. Chem. Journal*, xxxviii, p. 393, 1907; T. Brailsford Robertson: *Journal of Physical Chem.*, xiii, p. 469, 1909.

a refractive index, at the same temperature, of 1.3348. The refractive index of $\frac{N}{20}$ potassium hydroxide is 1.3338, and that of $\frac{N}{50}$ potassium hydroxide is 1.3335. Hence for the 1 per cent solution of ovovitellin in $\frac{N}{20}$ potassium hydroxide:

$$a = \frac{1.3351 - 1.3338}{1} = .00130$$

and for the 1 per cent solution of ovovitellin in $\frac{N}{50}$ potassium hydroxide:

$$a = \frac{1.3348 - 1.3335}{1} = .00130$$

From these investigations we may, I think, conclude that although the change in the refractive index of a solvent which is brought about by the solution of 1 gram of a protein in 100 cc. (the value of the constant a in the above equations) is not sufficiently different for different proteins to hold out much hope of our being able to estimate the *purity* of a given protein preparation from it, it is yet sufficiently different, for different proteins, to warrant the belief that its determination may afford valuable evidence regarding the *identity* of a given protein.

CONCLUSIONS.

(1) The refractive indices of solutions of ovomucoid in distilled water are connected with their concentrations by the formula:

$$n - n_1 = a \times c$$

where n is the refractive index of the solution, n_1 is the refractive index of the solvent, in this instance distilled water (1.3333 at 18°), c is the percentage concentration of the protein in the solution, and a is a constant which is numerically equal to the change in the refractive index of the solvent which is brought about by dissolving 1 gram in 100 cc. The same law has previously been shown to hold good for solutions of casein in various solvents.

(2) The value of a, in the above formula, for ovomucoid is 0.00160.

(3) For ovovitellin the value of a is 0.00130.

(4) For casein the value of a has previously been shown to be 0.00152.

THE ORIGIN OF THE BROWN PIGMENT IN THE INTEGUMENTS OF THE LARVA OF *TENEBRIO MOLITOR.*

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The question as to the cause of melanin formation has given rise to considerable investigation and much speculation, but it is only recently that the theories and the known facts have begun to coincide. Earlier investigators held that the melanin formation in red-blooded animals was due to products formed from the haemoglobin, but this failed to explain why iron was only a casual constituent of the melanin ash.

More recently the melanins have come to be looked upon as having an origin in the albumen, especially in those products with an aromatic or heterocyclic neucleus (tyrosin, tryptophan, etc)[1]. Compounds of this class when oxidized by artificial or naturally occuring oxidases undergo a series of color changes, ending in the case of oxidases of the tyrosinase type[2] with the deposition of a dark, insoluble, melanin-like body. Shortly after the discovery of tyrosinase by Bertrand[3] it was suggested that the oxidases, acting on various substituted aromatic or heterocyclic phenols, were the cause of melanin formation and this view has been gen-

[1] Schmiedeberg: *Arch. f. exper. Path. u. Pharmakol.*, xxxix, p. 65, 1897; Chittenden and Albro: *Amer. Journ. of Physiol.*, ii, p. 219, 1899; v. Furth and Schneider: *Beitr. z. chem. Physiol. u. Path.*, i, p. 229, 1902; Speigler, *ibid*, iv, p. 40, 1904; x, p. 253, 1907; v. Furth and Jerusalem: *ibid*, x, p. 131, 1907; and others.

[2] For literature of the oxidases see Bach: *Biochem. Centralbl.*, ix, pp. 1 and 73, 1909; and Kastle's "The Oxidases," Bull. no. 59, U.S. Hygienic Laboratory, 1910.

[3] Bertrand: *Compt. rend. acad. sci.*, cxxii, p. 1215, 1896.

erally accepted,[1] although in most cases the evidence is more reasoning from analogy than from direct observation.

Roques[2] noticed that during the metamorphosis of a Trichoptere (*Limnophilus flavicornis* Fabr.) the supply of tyrosinase in the body rose to a maximum just before pigmentation began in the nymph, and as pigmentation increased the tyrosinase correspondingly decreased until it was entirely absent in the fully pigmented beetle. From this observation he reasoned that tyrosinase acting on an oxidizible chromogen was the cause of the pigmentation.

In an earlier paper Dewitz[3] made a more complete demonstration leading to the same conclusion. In experiments with fly larva (*Lucilia Caesar*), he found that no oxidase was present in the very young larva, but that the quantity increased as the larva matured, although the larva remained perfectly colorless. When, however, the pupa was formed it became rapidly pigmented, the pigment appearing only when free oxygen was present, the coloration being inhibited by immersion in water, coating with olive-oil, etc. Dewitz also found that metamorphosis did not proceed in the absence of oxygen, although life was retained, and he concludes that pigmentation and metamorphosis in the fly larva are intimately related and are both due to the action of an oxydase (tyrosinase).

Phisilax[4] has recently investigated the color changes in the integuments of the cock-roach larva (*Phyllodromia germanica*). The larva when hatched from the egg is colorless, but in a short time it undergoes a series of color changes, passing through grey and brown until three hours after hatching the larva is entirely black. Phisilax shows that tyrosinase is present in the cockroach larva and concludes that "the cause of the change in color

[1] v. Furth and Schneider: *loc. cit.*; Dewitz: *Compt. rend. soc. biol.*, *liv*, p. 44, 1902 Gessard: *Compt. rend. acad sci.* cxxxviii, p. 586, 1903; Bertrand: *ibid.*, cxxxviii, p. 649, 1903; Durham: *Proc. Roy. Soc.*, lxxiv, p. 310, 1904; Bertrand: *Ann. Inst. Pasteur*, xxii, p. 381, 1908; Roques: *Compt. rend. acad. sci.*, cxlix, p. 418, 1909; Gessard: *Compt. rend. soc. biol.*, liv, p. 1304, 1902, etc.

[2] Roques; *loc. cit.*

[3] Dewitz: *loc. cit.*, and Gessard; *Compt. rend. acad. sci.* cxxxix, p. 644, 1904.

[4] Phisalix: *Compt. rend. soc. biol.*, lix, p. 19, 1905.

is due to the action of tyrosinase acting upon tyrosin,[1] these two substances existing in the embryo long before development and it is probable that they coexist in the egg or that they are deposited at the time of ovagenesis."

The observations recorded in this paper were made on the meal worm (larva of *Tenebrio Molitor*). The author has already shown[2] that the body filling of the meal worm contains two oxidases, a laccase-like enzyme and a powerful, insoluble tyrosinase, and that there is a chromogen present in the larva which, when acted on by the tyrosinase, gives a series of color changes ending in the deposition of a black, insoluble, melanin-like body.

The integuments of these larva are heavily marked with a brown pigment. Just after shedding, however, the larva is perfectly colorless excepting the tip of the mandibles and the claws, but the body rapidly colors, attaining its normal color intensity in 8 to 12 hours.

Several colorless larva were killed by the action of ether vapor and then decapitated to make sure that life was extinct. The bodies were then exposed to the air on moistened filter paper and the pigmentation was observed to proceed normally, although somewhat slower than in life, due probably, to the absence of circulation of the body fluids. When the same proceedure was repeated, replacing the air with carbon dioxide[3] or with nitrogen,[4] the body of the larva remained colorless until decomposition set in, although normal pigmentation occurred when the larva was removed from the action of carbon dioxide after twelve hours contact and exposed to the air.

These data indicate that the pigmentation is due to an oxydizing enzyme and, inasmuch as both tyrosinase and the laccase-like ferment are present in the meal worm, a number of colorless larva were dropped into water at 85° to 90° and allowed to remain

[1] No proof is given as to the identity of the chromogen which is present beyond the fact that an extract of the crushed larva will color when exposed to the air. The statement, therefore, that tyrosin is present is more theoretical than demonstrated by actual proof.

[2] Gortner: *Trans. Lond. Chem. Soc.* xcvii, p. 110, 1910.

[3] From marble and hydrochloric acid, washed through a sodium carbonate solution of pyro gallol and then through water.

[4] From sodium nitrite and ammonium chloride, washed through KOH, alkaline pyrogallol and water.

immersed for five minutes. Tests on a portion of these showed that tyrosinase had been completely destroyed, while the other oxidase was still very active. The remainder were exposed to the air and remained perfectly colorless until decomposition set in. It was also noticed that the colorless integument of the larva when washed free of body solids showed no tendency to color in the air, demonstrating that the enzyme, or chromogen, or both were to be found in the body-filling. Inasmuch as the coloration of the larva is not solid but a series of color-bands about the segments, it is suggested that, perhaps these bands are caused either by a more ready access to the air through pores in the integument or that the secretion of the chromogen is localized in these portions.

Further efforts were made to isolate the chromogen in a pure state, but these did not succeed. Thirty grams of the larva were ground in a mortar and washed into a liter of hot distilled water and the whole boiled for twenty minutes. The mixture was then filtered by suction and the precipitate discarded. The turbid filtrate was precipitated by basic lead acetate solution and filtered, giving precipitate "A" and solution "B."

Precipitate "A" was suspended in water and the lead removed by hydrogen sulphide. The filtrate was strongly concentrated, traces of sulphuric acid were removed quantitatively with baryta and a portion of the light yellow solution tested with insoluble tyrosinase[1] for the presence of the chromogen. No coloration was observed in 72 hours. This solution however contained a phenolic body which gave a deep red color with ferric chloride and a brown solution capable of dying silk an orange-brown shade when coupled with benzene diazonium chloride in alkaline solution.

Solution "B" was freed from lead by hydrogen sulphide and the filtrate evaporated to dryness, taken up in 30 cc. of distilled water, neutralized with sodium carbonate solution and tested with insoluble tyrosinase. On standing over night the solution had become very dark, showing that the chromogen was to be found here. Three cubic centimeters of concentrated sulphuric acid and 5 grams of phosphotungstic acid in 5 cc. of water were then added to the remainder of Solution "B," forming a heavy, greyish, crystalline

[1] For method of preparation see Gortner: *loc. cit.*

precipitate. This was filtered off (Precipitate "C"), leaving a colorless filtrate "D".

The phosphotungstic acid was removed from precipitate "C" by excess of baryta and the baryta quantitatively removed with sulphuric acid and the liquid concentrated. This gave no coloration with insoluble tyrosinase, gave a light red with ferric chloride and a deep red solution, dying silk, in an acid bath, an orange shade when coupled with benzene diazonium chloride in alkaline solution.

The phosphotungstic acid and baryta were removed from Filtrate "D" as above and the solution concentrated and neutralized with sodium carbonate solution. This contained the chromogen, as was shown by the darkening produced by the addition of insoluble tyrosinase, but the compound was not present in large enough quantity to determine its crystalline form, etc. A distinct coloration was produced, however, when tested with Millon's reagent. This solution also was capable of producing an azo-dye, deep red in alkaline solution and dying silk in acid bath a bright orange.

From the small quantity of the chromogen which is, apparently, present, it seems probable that it is secreted only as needed for the pigmentation which would require exceedingly small quantities,[1] while the oxydase is constantly present in relatively large amounts. The view that the chromogen is secreted only as needed for pigmentation is also upheld by the fact that the pupa is colorless when the larval skin is first shed and only changes color slightly on exposure, becoming a very light brown shade on those spots which are heavily banded in the larva. Apparently, the secretion of chromogen ceases when the larva changes to the pupa for the chloroform water extract of crushed pupa (having been ten days in the pupa state) allowed to stand over night does not darken to any appreciable extent, although tyrosinase is still shown to be present by the addition of a chromogen (tyrosin). When the adult is formed the secretion of chromogen is resumed, as shown by the coloration of the extract of crushed beetles in the air without added chromogen and also by the fact that pigmentation is, now,

[1] The total weight of the integuments of the 30 grams of larva was only approximately 0.4 gram and only a very small percentage of this coating is pigment.

resumed, proceeding through a light brown to a black, due to the concentration of the brown pigment.[1]

SUMMARY.

(1) The pigmentation of the integuments of the larva of *Tenebrio molitor* is the result of the interaction of an oxydase and a chromogen.

(2) The process of coloration has been shown to be an enzyme process, in that it is capable of proceeding in the absence of life.

(3) Pigmentation which has been halted by the removal of oxygen proceeds normally when the inhibitor (carbon dioxide, nitrogen, etc.) is removed.

(4) The chromogen, like tyrosin,,is not precipitated by phosphotungstic acid.

(5) The chromogen is apparently secreted only as needed for pigmentation and is therefore present in exceedingly small amounts at any one time.

(6) Tyrosinase is present in both the pupa and the beetle, but the chromogen is apparently lacking in the pupa stage, the only stage without pigmentation.

(7) A phenolic body is present in the larva of *Tenebrio molitor*, as are, also, bodies capable of uniting with diazo compounds to form azo dyes.

[1] Davenport and Davenport (*Amer. Naturalist*, xliii, p. 193, 1909) have shown that most "black" hair, etc. is due to the presence of a sepia pigment in strong concentration.

AUTOLYSIS OF FERTILIZED AND UNFERTILIZED ECHINODERM EGGS.

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The present paper is concerned in large part with the increase in the soluble nitrogen of unfertilized and fertilized eggs of the sea urchin when allowed to undergo aseptic auto-digestion. Because of the small number of experiments, conclusions are, in the main, only tentative, and are perhaps merely suggestive of more clearly defined lines for further investigation.

The eggs of *Arbacia* were used in all the experiments cited. They were collected and the autolyses started while one of us was at the Beaufort laboratory of the U. S. Bureau of Fisheries last summer. Two experiments were finished at Beaufort, one at Woods Hole[2] and the rest in the physiological laboratory of St. Louis University. As in the work on catalase of eggs recently published by one[3] of us "the eggs were taken from the females and allowed to settle several times in renewed, large quantities of sterile sea-water. They were thus freed as well as possible from immature eggs, body cells and other material. A large volume of eggs suspended in sea water was divided into a number of equal lots. Care was taken to have these uniform by constant agitation while the measuring was going on." Since there was a

[1] To the Commissioner, the Hon. George M. Bowers and the Directors of the Laboratories, Mr. H. D. Aller and Dr. F. B. Sumner, respectively, our cordial thanks are due.

[2] The analyses for this experiment were made by Dr. D. D. Van Slyke, to whom we desire to record our obligation.

[3] Lyon: The Catalase of Echinoderm Eggs before and after Fertilization, *Amer. Journ. of Physiol.*, xxv, p. 199, 1909.

general similarity in the experiments, the following protocol is typical of the general procedure:

July 23, 1909, eggs from a large number of *Arbacia* females were washed three times in sterile sea water. 50 cc. of this suspension of eggs, filtered free from sea water and dried, weighed 0.44 gram. The remaining eggs were placed in previously sterilized bottles Nos. 1–15, 5 cc. of chloroform being added later to each bottle.

No. 1, 100 cc. suspension of unfertilized eggs plus 10 cc. 3 per cent acetic acid.
No. 2, 100 cc. suspension of unfertilized eggs plus 10 cc. 3 per cent acetic acid, boiled.
No. 3, Filtrate from 100 cc. suspension of unfertilized eggs plus 10 cc. 3 per cent acetic acid, boiled and filtered at once.
No. 4, 100 cc. suspension of unfertilized eggs (without acid).
No. 5, " " " " " " plus 10 cc 3 per cent Na_2CO_3.
Nos. 6 and 11, like 1 except eggs *fertilized* 50 and 120 minutes respectively.
Nos. 7 and 12, " 2 " " " " " " " "
Nos. 8 and 13, " 3 " " " " " " " "
Nos. 9 and 14, " 4 " " " " " " " "
Nos. 10 and 15, " 5 " " " " " " " "

In other words, Nos. 1, 6 and 11 autolyzed in acid; 4, 9 and 14 in neutral and 5, 10 and 15 in alkaline solution; 2, 7 and 12 were boiled but the precipitate left in contact with the liquid; 3, 8 and 13 boiled and filtered at once. All bottles were sealed and placed in a shaking machine in the engine room for 8 days, then packed for transportation. Autolysis continued over three months in this experiment, the analyses being made at St. Louis.

After undergoing autolysis in the chloroform water for varying periods of time the suspensions of eggs were boiled, those that had acted in neutral or alkaline solution being first acidified slightly with acetic acid. The insoluble material was in each case filtered off and discarded; the filtrate was made up to a definite volume, and the nitrogen determined in an aliquot by the Gunning-Kjeldahl method, using 10 cc. of concentrated sulphuric acid and 0.7 gram of mercury in the digestion. The ammonia was determined as usual by distillation into standard sulphuric acid.

In the present experiments no total nitrogen, and but three dry matter determinations were made. It is possible, therefore, to compare the several experiments only in so far as the individual determinations of each tend in a definite direction. Because

of this the nitrogen results are reported in terms of their equivalents in cubic centimeters of $\frac{N}{10}$ sulphuric acid.

DISCUSSION OF RESULTS.

Table I includes the results of seven experiments in which the nitrogen changes were studied. It will be seen that in the case of both fertilized and unfertilized eggs allowed to autolyze in acid media there is a consistent increase in the soluble nitrogen over that in the boiled controls: this is not evident or but slightly so where eggs were autolyzed in neutral or alkaline media. Although the data indicate that the process of fertilization and the interval of time after the sperm is added until the chloroform is added, exercise little, if any, influence on the autolysis, the results are not constant enough to warrant any conclusion being drawn. Experiments using denser suspensions of eggs are needed to settle these questions. Especially should rhythmical changes of autolytic power corresponding to the different phases of cleavage be looked for since it is known that the physiological properties of eggs vary so markedly during each cleavage.

All the controls were boiled in acid solution. In experiments III–VI double controls were run; in one set (boiled, unfiltered) the eggs were left in suspension after boiling, the filtrates being obtained later and analyzed with those of the autolyzed samples. The other controls in these experiments (boiled, filtered) were filtered immediately after boiling. The unfiltered controls show a constant increase in soluble nitrogen over that of the filtered samples.

In Table II are data obtained on sperm autolyzed under the same conditions as were the ova. Contrary to the results with eggs, however, the sperm shows a large increase in soluble nitrogen in neutral or alkaline solution, whereas in acid solution there is no increase of nitrogen over that of the boiled controls.

It should be stated here that possible contamination of the samples by bacteria was controlled in the first three experiments by bouillon cultures and in the rest by plating on agar-agar. Our thanks are due Dr. J. W. Marchildon for making these examinations. Growths were obtained only in the case of the eggs and sperm autolyzed in an alkaline medium. This would seem to

TABLE I.

Results in terms of $\frac{N}{10}$ H_2SO_4	Autolyzed (acid).	Autolyzed (neutral).	Autolyzed (alkaline).	Boiled (unfiltered).	Boiled (filtered).
Experiment I: begun July 18, 1909; autolyzed 2 days.					
Unfertilized..........	3.72				3.40
Fertilized 8′........	3.93				3.61
" 15′........	4.05				3.74
" 25′........	4.20				3.62
" 45′........	4.00				3.31
Experiment II: begun July 19, 1909; autolyzed 7 days.					
Unfertilized..........	4.18				3.80
Fertilized 5′.........	3.99				3.84
" 30′.........	4.09				4.38
Experiment III: begun July 20, 1909; autolyzed 34 days.					
Unfertilized..........	23.84			11.32	10.60
Fertilized 10′.........	23.51	15.34		13.11	12.56
" 30′.........	22.69	15.90		13.28	12.90
Experiment IV: begun July 23, 1909; autolyzed 3 months.					
Unfertilized..........	7.90	5.00	5.16	6.74	6.86
Fertilized 50′........	8.28	5.56	5.41	7.65	7.45
" 120′........	8.05	5.40	5.38	7.24	6.61
Experiment V: begun July 24, 1909; autolyzed 3 months.					
Unfertilized..........	14.28	12.00	11.59	12.10	11.38
Fertilized 25′.........	15.00	12.20	15.06	13.08	12.83
Experiment VI: begun July 26, 1909; autolyzed 3 months.					
Unfertilized..........	15.83	10.76	10.76	12.10	11.90
Fertilized (Time?)...		11.59		10.96	10.76
Experiment VII: begun July 30, 1909; autolyzed 3 months.					
Unfertilized..........	36.44				
Fertilized 60′.........	35.74			25.50	

nullify the results obtained, especially on the sperm; but the fact that the sperm in neutral medium which was found to be sterile autolyzed to about the same extent as in an alkaline medium, makes it probable that bacterial contamination in the present experiments caused little or no change in the amount of nitrogen rendered soluble.

An attempt was made to differentiate between the forms of nitrogen in the filtrates of autolyzed and control samples. Lack of material, however, prevented more than one experiment of this kind which was done on the material of experiment VII. The reagents used were trichloracetic acid and tannic acid. The former, according to Vernon, precipitates native proteins only, while the latter which was used in 12 per cent solution throws out presumably all but amino-acids. In the case of trichlor-

TABLE II.

(Autolyses continued three months.)

Results in terms of $\frac{N}{10}$ — H_2SO_4	Autolyzed (acid).	Autolyzed (neutral).	Autolyzed (alkaline).	Boiled (unfiltered).	Boiled (filtered).
Sperm I............	15.56	33.05	32.65	20.08	17.53
" II............	9.85	17.75	19.41	10.76	9.10

acetic acid 2 cc. of the melted reagent were added to 100 cc. of the shaken suspension of eggs previously heated to boiling. The solutions were immediately filtered and washed, and nitrogen determined in the filtrates. Where the nitrogen precipitable by tannic acid was determined, 10 cc. of the reagent were added in the cold to 100 cc. of the suspension of eggs and allowed to stand over night. Each sample was then filtered, washed thoroughly and the nitrogen determined in the filtrate. To a second set of filtrates from tannin precipitation were added while boiling 10 cc. of a 10 per cent solution of phosphotungstic acid in 4 per cent hydrochloric acid. Not the slightest cloudiness appeared, however, so the nitrogen content was not determined.

The data obtained in the differentiation of nitrogen are given in Table III. The results with trichloracetic acid are not interpretable and will not be discussed. In the case of the tannic acid, however—allowance being made for but the single experi-

ment—the results indicate that there is no more proteid nitrogen in autolyzed samples than in the boiled controls. This, if true, leaves but two explanations for the marked autolysis, shown especially in experiments III and VII—the soluble nitrogen was mainly in the "amid" form, or had come from nuclein or lecithin compounds. That the first was not true was shown by distilling with magnesium oxide an aliquot of each of the filtrates in experiment III. Only traces of nitrogen were obtained from the autolyzed samples and none from the boiled controls. During the distillations, moreover, there was noted in the receivers

TABLE III.

Results in terms of $\frac{N}{10}-H_2SO_4$	Nitrogen in filtrate after boiling in acetic acid solution.	Nitrogen in filtrate, after boiling with trichloracetic acid.	Nitrogen precipitated by trichloracetic acid.	Nitrogen in filtrate after precipitation by tannin.	Nitrogen precipitated by tannin.
Unfertilized (Autolyzed)	36.44	35.89	.55	32.53	3.91
Fertilized 60′ (Autolyzed)	35.74	35.26	.48	32.16	3.58
Fertilized Control (Boiled)	25.50	26.90	−1.40	21.25	4.25

TABLE IV.

Results in milligrams of $Mg_2P_2O_7$.	Autolyzed (acid).	Autolyzed (neutral).	Boiled (unfiltered).	Boiled (filtered).
Unfertilized	14.0		9.9	9.6
Fertilized 10′	13.6	13.6	9.8	11.2
" 30′	13.2	13.6	10.0	9.8

connected especially with the autolyzed samples the characteristic fishy odor of trimethylamine. It thus seemed not improbable that some at least of the autolyzed nitrogen had come from the breaking up of lecithins, and that the trimethylamine had been produced from one of the products, choline, by distillation with the magnesia.

One hundred cubic centimeters of each filtrate in experiment III remained, and a total phosphorus determination was accordingly made on each portion after digestion with 10 cc. of concen-

trated sulphuric acid. Table IV gives the results of this series in milligrams of magnesium pyrophosphate found. Though the amounts are small and the differences might be held to come within the limits of experimental error, there is nevertheless a consistent parallelism in this experiment between the autolysis of nitrogen and phosphorus with the exception that the phosphorus autolyzed in neutral medium is approximately equal to that in acid medium, while, as has been seen, there is a greater autolysis of nitrogen in acid than in neutral solution. These points need, of course, further confirmation.

SUMMARY.

(1) In all experiments (7), eggs of *Arbacia*, both fertilized and unfertilized, show a consistent increase in soluble nitrogen over that in boiled controls when allowed to undergo autolysis in chloroform water for periods varying from a few days to three months. This increase is noted in acid solution, with none or but little in neutral or alkaline media.

(2) Fertilization seems to exert but little or no effect on autolysis.

(3) In controls unfiltered until the autolyzed samples were analyzed, there is a constant increase of soluble nitrogen over the controls that were filtered immediately after boiling.

(4) One experiment indicates that not more than one-sixth of the soluble nitrogen from autolyzed or control eggs is of proteid origin.

(5) Distillation with magnesium oxide shows almost no "amid" nitrogen in filtrates from autolyzed or control eggs.

(6) A single experiment indicates that in fertilized and unfertilized eggs the autolysis of phosphorus runs parallel with that of nitrogen. An exception noted is that the autolysis of phosphorus is about equal in acid and neutral media, whereas the increase in nitrogen is confined to eggs digesting in acid solution.

(7) Two experiments with sperm show a large increase in soluble nitrogen when autolyzed in *neutral* or *alkaline* media, with but little or no autolysis indicated in acid solution.

We hope to continue work along this line during the coming summer.

STUDIES OF THE INFLUENCE OF VARIOUS DIETARY CONDITIONS ON PHYSIOLOGICAL RESISTANCE.

I: THE INFLUENCE OF DIFFERENT PROPORTIONS OF PROTEIN IN THE FOOD ON RESISTANCE TO THE TOXICITY OF RICIN AND ON RECUPERATION FROM HEMORRHAGE[1].

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Carbohydrates and fats are essentially sources of energy for the animal body, whereas proteins provide most of the material for the animal constructive and reparative processes. It is important that the three types of primary organic food substances should be so adjusted, in kind and amount, as to furnish liberal supplies for all the necessary energy transformations and for all the needed processes of construction and repair. This qualitative and quantitative coördination is particularly important with respect to protein, because of the urgency of the body needs for nitrogenous construction and repair material; because protein may supply the required energy, when the available amounts of carbohydrates and fats are reduced; and because an immoderate excess of protein may give rise to disadvantageous nutritive conditions Except during the period of adolescence, the body does not, under

[1] A preliminary report of this work was published in the *Proceedings of the Society for Experimental Biology and Medicine*, vi, p. 61, 1909; also *Proceedings of the American Society of Biological Chemists*, 1909, i, p. 195; this *Journal*, vi, p. xlviii, 1909.

The work has been frequently interrupted by researches in other directions and has been beset by unusual experimental difficulties. Although planned to be the first of a series of investigations, in point of publication it is the second from this laboratory on the general subject stated above. See Dissertation by Welker, Columbia University, 1908. It is Dr. Gies' intention to continue investigations along these lines.

ordinary circumstances store up a reserve supply of protein, as it does in the cases of carbohydrates and fats; and the excess of ingested nitrogen over the immediate demand is usually excreted with remarkable promptness. Protein food is primarily needed to replenish tissue waste, but normal tissue anabolism and catabolism do not appear to require the quantity of nitrogenous food that is ordinarily consumed. The recognition of this tendency to excess in the protein portion of our common dietaries has occasioned numerous studies, during the past few years, on the sufficiency of diets low in protein contents.

There are certain aspects of this last question, not at first sight evident, which require more consideration than they have received. While the proteins of all tissues are alike in consisting very largely of amino acid radicals linked together in complex molecular arrangements, analogous tissues from different species of animals are not alike in their qualitative protein contents, as is shown by precipitin reactions and, in many cases, by various methods of chemical analysis. Many inherent differences among proteins are dependent upon the kinds, proportions, and arrangements of their various amino-acid radicals. For each animal species (and perhaps even for each individual) a certain definite amino-acid *yield* on hydrolysis is characteristic of every organ. The primary function of cellular as well as intestinal digestion, as it is conceived today, is the distintegration of proteins, e.g., serum albumin, into their amino-acids so that reconstruction into *specific cellular* proteins may ultimately take place. In this connection mention may be made of Abderhalden's attempt to vary the glutamic acid yield of the blood of a horse by feeding gliadin, which is rich in the radicals of this acid; but the blood composition remained stationary in this respect.[1]

With this conception of the specificity of organized proteins in mind, it is evident that the question of food requisites in protein must be restated; the nutritive value of a protein depends not on its nitrogen content but on its amino-acid *yield* and on the qualitative and quantitative accord of such acid *yields* with the amino-acid *requirements* of the tissues to be nourished. The

[1] Abderhalden: *Centralb. f. d. ges. Physiol. u. Pathol. d. Stoffwechsels*, i, pp. 225 and 497, 1906.

nutritive value of gelatin is an excellent illustration of this point. It has been known for years that gelatin, although a protein *rich in nitrogen*, and theoretically a valuable one, will not maintain mammalian life, unless other protein matter is fed with it. The reason for this inadequacy is that gelatin is poor or lacking in certain of the amino groups required for vital bioplastic constructions. It may often be necessary, when the variety of food is greatly restricted, to consume relatively large amounts of a given protein in order to secure those particular "construction units" which the body happens to require for reconstructive processes. Then again, the differences in chemical composition of the organs of the body make it evident that certain radicals which occur only in traces in ordinary food materials are utilized by some of the organs to an exceptional *quantitative* extent (e.g., aromatic radicals by the suprarenal gland). This fact suggests an inevitable local waste of by-products as the local imperative demands are satisfied. The interesting experiments of Willcock and Hopkins,[1] who noted an improved condition of health and prolonged life in mice fed on a zein diet when tryptophan was added to the food, are suggestive in this relation.

The general point I wish to emphasize in this connection is that many other factors are concerned in an estimation of the protein requisites of an individual, besides the simple gross nitrogen elimination and a replacement of this nitrogen in the forms of any digestible protein equivalents.

In its pathological relations, the effects of excess or of deficiency of nitrogenous foods have been considered from many standpoints. From the time of Sydenham excess in this respect has been held to be a factor in the etiology of gout; and, more recently, in relation to the various cardiovascular and renal disorders. On the other hand attention has been called to the incidence of tuberculosis among vegetarians, the relative immunity to this disease of the carnivora, and the efficacy of "zomo" therapy in tuberculous individuals.[2]

From a broad point of view, the general nutrition of the individual, irrespective of the kind of food, bears some relation to

[1] Willcock and Hopkins: *Journal of Physiology*, xxxv, pp. 88–102, 1906.
[2] Hare: *Practitioner* (London), xxiii, pp. 179–190, 1906.

his viability and his susceptibility to disease. The experience of clinicians that, in general, the prognosis with fat persons suffering from pneumonia is good and from typhoid bad; and that the reverse is true with those of slender build, illustrates the difficulty of ascertaining the facts in this regard. In a paper presenting elaborate life insurance statistics, Symonds has shown the decrease in expectancy of life among individuals who are over average weight, but these statistics, valuable as they are, will not support deductions in regard to food.[1]

Of the laboratory experiments upon man, those that have had in view the proof of some special theory must be examined with particular care and proper allowance made for the psychological element, as Albu has pointed out.[2]

In so far as nitrogenous food is concerned, the habits of the native tribes of India present experimental conditions that are not to be secured in any laboratory, because the food of these people is of their own selection. Their diet is poor in protein, the restriction being in accord with a religious law in operation for many generations. Among these people, therefore, any ordinary obstacles to food adaptations have long since been overcome. With these facts in mind, the studies of McCay on the protein diets of the Bengali are of special interest.[3]

McCay finds that the nitrogen ingest is highest among the wealthy classes represented by the students, and lowest among the poor laborers. The average daily nitrogen elimination in the urine, for 124 students, was 6.05 grams and for the "domes" or laborers, 5.96 grams. The results of numerous analyses indicate that the protein intake (average for all classes) amounts to 37.5 grams per day or 32 per cent of Voit's standard; or, figured on a per kilo basis, 0.11 gram of nitrogen per kilo.

In commenting upon his results McCay asserts that the Bengali are, compared with Europeans, poorly developed, that they are physically weak and that the incidence of renal and cardiovascular diseases is so great as to suggest chronic malnutrition

[1] Symonds: *Medical Record*, lxxiv, pp. 389–393, 1908.

[2] Albu: *Berlin. klin. Wochenschr.*, xxxv, p. 111, 1898.

[3] McCay: *Scientific Memoirs of Officers of Medical and Sanitary Departments of the Government of India*, Calcutta, 1908.

as a cause. Diabetes is also a very common disease compared with its occurrence in Europeans.

Such facts emphasize the necessity of practical consideration of the subject of protein nutrition, as well as the desirability of answering such questions as this: Has the *amount* of protein food any direct relation to *physical stamina*, as interpreted in terms of immunity, or ability to overcome toxic agents, or ability to regenerate and repair tissue losses? It is to this special part of the larger question that this investigation has been directed. The particular problems with which this paper deals may be formulated as follows: Is the *amount* of protein intake an important factor in the resistance of animals to certain toxins, and to tissue loss as occasioned by hemorrhage, or is the influence of *quantity* of ingested nitrogen relatively minor under such conditions, as compared with other factors which may be designated collectively as individual idiosyncrasy?

EXPERIMENTS WITH RICIN.

INTRODUCTION. In endeavoring to reproduce in dogs those conditions which characterize acute infectious disease, one is confronted with a difficult problem. Dogs are not easily infected with the common bacteria that are pathogenic for man. It is usually necessary to produce a *locus minores resistentia* by inflicting some serious trauma in order to get such an organism as streptococcus to grow and produce an infection. Consequently such bacteria are of little use in metabolism work on dogs, especially if carefully executed controls are a prime requisite. For these reasons, and at the suggestion of Dr. Flexner, ricin was selected as the toxic substance for use in the first series of these experiments.

In its action on animals ricin produces many phenomena that are clinically like acute infections; the fever, prostration, and cardio-vascular manifestation are closely analogous to the profound toxemic conditions caused in humans by typhoid and pneumonia. These symptoms are in part due to the very marked hemolytic action exerted by ricin.

There are, however, very serious disadvantages in the use of ricin in metabolism work, as I have found to my cost. The first

of these is that this substance is so exceedingly toxic that when the endeavor is made to use a dosage just under the lethal, sufficient leeway cannot be allowed for individual variation in the animal inoculated. Moreover, unavoidable error results from fixing the dosage on the weight of the dog, since with dogs that are fat such dosage must be relatively greater than with dogs that are lean.

The second disadvantage in the use of ricin arises from its developing, in inoculated animals, an immunity even after one injection and this immunity precludes accurate control observations on the same animal.

In this part of the study the object was to compare the behavior of two sets of animals under variously induced pathological conditions, one series to be fed with as much ordinary nitrogenous food as they would consume, the other to be given as little protein as would maintain the animal in an apparently normal condition.

Dogs were employed. In selecting the dogs, pairs that were as much alike as possible were taken; for this reason, as will be noted, more dogs of a fox terrier type were used than of other breeds. Of course the ideal condition would be to compare in each experiment two animals from the same litter but that plan was not feasible.

In each case the dog was fed on the diet regularly in use in this laboratory and composed of lean beef hash, cracker meal and lard. The meat was prepared in large amounts and kept in a frozen state until used. Samples of meat and cracker meal were analyzed for the N content. At the commencement of metabolism work on each dog, a diet was given in which the various constituents were present in the proportions which we have found are adequate for dogs. Then the meat was increased or decreased in amount according to the purpose of the experiment, by small daily increments, until the desired nitrogen plane was attained.[1] Such a slow and deliberate process of changing the

[1] No attempt was made in these experiments to preserve equal caloric values in the food by simultaneous compensatory additions of one ingredient, e.g., carbohydrate, when the proportion of another constituent of the diet, e.g., protein, was diminished. While it was recognized, of course, that the maintenance of the same number of calories in the food

amount of protein in the diet prevents gastro-intestinal disturbances as well as strained nutritional readjustments, and also allows the observer, by frequent analyses, to feel his way, as it were, to the desired metabolic condition of the animal. In all cases it was part of the plan of the work to keep the animal on a high or low nitrogen plane for a considerable period *before* the pathological phase was brought into the experiment. Such a course is obviously not only desirable but absolutely essential, if any valuable deduction pertaining to analogous human conditions is to be made from such experiments. Whatever influence a definite nutritional state may exercise as a factor in the immunity of human beings from, or their predisposition to, disease is ordinarily not manifested at once but, as a rule, is evidenced only after long continuation of that metabolic condition.

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throughout each experiment would be desirable, yet it was obvious that this could not be achieved without introducing influences quite as undesirable as the caloric fluctuation itself. In each experiment there was, therefore, an increase or decrease in the total caloric value of the food, depending on whether the protein was increased or diminished. On the basis of its determined nitrogen content, the meat caloric value was one calorie per gram. This figure agrees with those in Atwater's tables for the same parts of beef (top round). The subjoined table gives the caloric values of the diet of each dog *at the commencement of* each experiment (variations in the diet after the beginning of each experiment are noted in each description of the work):

RICIN EXPERIMENTS	MEAT: CALORIES	CRACKER MEAL: CALORIES	LARD: CALORIES	TOTAL: CALORIES	CALORIES PER KILO OF BODY WEIGHT
Experiment I	45	140.4	211.2	396.6	66
Experiment II	355	234.0	264.0	853.0	55
Experiment III	42	97.5	159.5	299.0	56
Experiment IV	401	148.2	237.6	786.8	71
Experiment V	150	174.5	220.0	544.0	41
Experiment VI	400	156.0	237.6	793.6	77
HEMORRHAGE EXPERIMENTS					
Experiment I	64	124.8	220.0	408.8	54
Experiment II	200	97.5	158.4	455.9	76
Experiment III	500	171.6	290.4	962.0	75
Experiment IV	50	109.2	184.8	344.0	57
Experiment V	40	123.2	220.0	383.2	46
Experiment VI	194	140.4	237.6	572.0	62

In some of these experiments the preliminary periods were not continued so long as was desired, because of the heavy expense connected with the feeding.

In the ricin experiments the dosage was computed on a kilo basis and the same dose per kilo was given to all the animals in this series. Differences in reaction to the toxin were estimated as accurately as possible from clinical aspects, the metabolic results serving as accessory data. Variations in nitrogen metabolism are hardly capable, at present, of interpretation in terms of degrees of physiological resistance.

In these inoculation experiments ricin, in known amounts, was dissolved in 10 per cent sodium chloride solution and then diluted with water until each cubic centimeter contained a certain desired amount of the toxin. By a series of preliminary observations on dogs, it was found that a dose of 1.2 mgm. per kilo of body weight invariably produced very severe symptoms but in only one dog out of seven resulted in death. Doses of 1 mgm. per kilo resulted in no fatalities in the preliminary experiments. That amount was therefore considered the maximum non-lethal dose.

FIRST RICIN EXPERIMENT. *First part. Subcutaneous injection of ricin in a dog on a low plane of protein nutrition.*

The animal was a fox terrier bitch, weighing 7.6 kilos. Her dieting was begun on August 22, 1905. The daily food at the beginning consisted of 144 grams of meat, 36 grams of cracker meal, and 24 grams of lard, with 8 grams of bone ash and 300 cc. of water. This mixture contained 5.82 grams of nitrogen, an amount equal to 0.76 gram of nitrogen per kilo. The quantity of meat in this diet was reduced three grams per day successively until October 9, when only 24 grams of meat were allowed daily until October 21. The other food ingredients were unchanged in amount. This diet contained 1.62 gram of nitrogen (0.22 gram per kilo), while the average daily nitrogen excretion amounted to 1.75 gram. The weight of the dog during this period fell from 7.6 kilos to 6.97 kilos. From October 9 to 21 the daily amount of meat in the food was held at 24 grams but as the animal lost weight (from 6.97 to 6.67 kilos), the quantity of meat was cumulatively increased gradually to 45 grams daily. On this allowance the weight of the dog remained practically stationary from October 30 to November 8. The daily nitrogen elimination averaged 1.82 gram during this period, while the quantity of ingested nitrogen amounted to 2.29 grams.

It was decided to decrease again the amount of protein in the diet in order to ascertain whether nitrogenous equilibrium could be secured on an even lower plane. To this end the quantity of meat was diminished 3

grams per diem successively until the daily allowance (November 23) was 15 grams, when the total amount of ingested nitrogen was 1.197 gram or 0.18 gram per kilo. These dietary conditions were maintained from November 23, to December 2, the dog's weight decreasing from 6.43 to 6.21 kilos in these nine days and the daily nitrogen excretion averaging 1.796 gram. This average daily loss of 0.599 gram of nitrogen, as indicated by the excess of excretion over ingestion, represents an amount of pure protein approximately equal to the average daily loss in weight of this dog during these nine days.

Inasmuch as it was evident that the animal could not be kept in proper health under the above mentioned conditions, the quantity of meat in the diet was cumulatively increased a few grams daily from December 2 until the allowance per day was 45 grams (December 11). Even after this increase in its nitrogenous food, the animal continued to lose in weight for fourteen days, December 16; then the weight slowly returned to a practically constant figure, fluctuating between 6.25 and 6.3 kilos from December 26 to January 8 (1906). On the latter date approximate nitrogenous equilibrium had been established, the average daily quantity of ingested nitrogen amounting to 2.196 grams (0.35 gram of N per kilo) and the average daily excretion of nitrogen equalling 2.29 grams. At this time the dog was very thin but in no other respect than lack of flesh could anything abnormal be detected. She was lively and playful, and as restless as fox terriers are usually.

The conditions being suitable, on January 9 the first inoculation of ricin was made, 6 milligrams of the toxin being used and the injection made in the soft tissue of the inner aspect of the thigh. No immediate symptoms were manifested other than pain due to the irritating toxin. The pain seemed to disappear in the course of half an hour. The following notes were made during this period:

January 9, 1906. 10:30 p.m. Five hours after inoculation. Dog appears quite sick; pulse rapid, 140 to the minute, temperature 105°.

January 10, 8:30 a.m. Condition same as last night. Pulse 160, respiration 12 to the minute, temperature 104.4°. *10:30 a.m.* Dog has eaten all her food. Pulse 134, temperature 104.4.° *12 m.* Has a chill. *5 p.m.* Refuses to move, respirations are labored. Temperature 104.4°. *9 p.m.* Pulse 168, respiration 18, temperature 104.2°. No change in general condition. *11 p. m.* Condition unchanged.

January 11, 8 a.m. Respirations slow and deep like those in Kussmaul's air hunger, pulse weak and irregular, often intermits. Temperature 103.9°. There was no change in the symptoms during the day except a slow falling of the temperature to 103.2° at 10 p.m. The peculiar deep, respiratory movements continued and at that time I believed the dog would die during the night.

January 12, 8:30 a.m. Seems a little less apathetic. Respiration still labored, pulse 140, temperature 102.2.° *3 p.m.* Ate all her food eagerly. Temperature 101.5°.

January 13, 10 a.m. Much improved. The site of inoculation is much inflamed and swollen.

Thereafter recovery was uneventful and, aside from the wound caused by sloughing at the site of inoculation, there where no noteworthy manifestations of abnormal condition.

The effects of the toxic action of ricin on the metabolism are shown in Table 1. The marked increase in the amounts of excreted nitrogen and sulphur following the inoculations would be expected when one considers the powerful hemolytic action of this toxin.

Second part. Subcutaneous injection of ricin in a dog on a high plane of protein nutrition. Inasmuch as these studies were intended to be comparative it appeared of some interest to ascertain how this dog would react to a second inoculation of ricin after having been *liberally* fed for some time.

To this end the amount of meat in the diet was cumulatively increased 10 grams daily from 45 grams commencing January 21st. By March 8th, the dog received 400 grams of meat daily, with cracker meal, lard, bone ash and water in the same amounts as those given previously throughout the experiment. This daily diet (containing 15.21 grams of N) was then maintained until after the second inoculation with ricin on April 16. There was a gain of 3 kilos in weight between January 21 and April 16. At this time the animal was in splendid physical condition. In appearance she was in marked contrast to that of three months previous.

April 16, 5 p.m. Six mgm. of ricin, the same dose as in first inoculation, were administered.

April 17, 9 a.m. Dog is quiet and acts as if cold, but does not look sick. Temperature 104.2°. No respiratory distress.

April 18, 1:30 a.m. Has eaten all her flood. No change in condition. *10 a.m.* Acts less sick than yesterday; is quiet, chiefly on account of soreness of the leg, apparently. Temperature 103.4°. *12:30 p.m.* Improvement continues, stands up and wags tail when spoken to.

April 19. Aside from lameness in the leg, the dog appears quite well. Temperature 102°.

Recovery thereafter was uneventful. The recorded metabolic data are given in Table I. It was quite evident that the dog was much less sick as a result of the second inoculation of ricin than as a result of the first. For this fact two explanations seemed evident. Either the improved condition of the animal after three months of liberal feeding had rendered her more resistent or else the first inoculation had given her a certain degree of immunity. In either case it seemed probable that a third injection of ricin, after a proper period of restricted protein feeding, would lead to a correct interpretation of the facts, so far as any influence of quantitative protein dieting was concerned.

Third part. Subcutaneous injection of ricin in a dog on a LOW *plane of protein nutrition.*

The diet was again gradually reduced in its meat content until the daily allowance of food contained 2.43 grams of nitrogen (0.30 per kilo) on May 31 (52 grams of meat). This diet was maintained thereafter through the third part of the experiment in an endeavor to approximate the conditions associated with the first inoculation.

July 16. A third inoculation was made with the original dose of ricin (6 mgm). There was hardly any noticeable reaction. There was no rise in temperature, and the next day (July 17) the animal appeared as well as ever.

These negative results indicate strongly that the diminished effects of the second inoculation were due to a certain degree of immunity resulting from the first injection, rather than to the improved condition of the animal.

In the remaining experiments with ricin, comparative effects were obtained in different animals under nearly identical conditions, rather than under different conditions in the same animal. The results of this particular experiment made such observations necessary. Unfortunately neither method is perfectly satisfactory in experiments of this kind.

Effect of Exercise on the Dog on a Low Plane of Protein Nutrition.

From what had been observed during the various parts of this experiment, it seemed clear that this dog preserved excellent health on a diet containing comparatively little nitrogen, the only very evident effect of the meagre diet being marked emaciation. It was necessary, of course, to confine the animal in a metabolism cage throughout the experiment, but she appeared to be as active as the well fed dogs thus confined. But the question naturally arose: Would a dog fed on a diet low in its protein content be normally active if allowed to run about freely?

The dog used in this experiment appeared to be in perfect health on July 17, 1906, after her last inoculation. She was then put on the diet which in December and January appeared just adequate for her needs on a low plane of protein nutrition. During that time she had been in approximate nitrogenous equilibrium on a daily allowance of 2.19 grams of nitrogen per day. Beginning with July 17, she was given 43 grams of meat daily (with 36 grams of cracker meal, 24 grams of lard, and 8 grams of bone ash and 300 cc. of water as theretofore), the nitrogen of the food

TABLE I.

First Ricin Experiment. *A.* First Part. Low *plane of protein metabolism.*

a. Period immediately before *the* first *injection of ricin.*

Date 1906 Jan.	Body weight kilos.	Volume of urine cc.	Sp. gr. of urine 1.0xx.	N of urine grams.	N of feces gram.	Total N grams.	S of urine gram.	S of feces gram.	Total S gram.	Weight of dry feces grams.
3	6.30	345	09	2.40	0.09	2.49	0.17	0.02	0.19	8
4	6.28	370	08	2.57	0.04	2.63	0.18	0.03	0.21	5
5	6.28	365	08	2.54		2.54	0.18		0.18	
6	6.24	375	09	2.16	0.09	2.25	0.19	0.04	0.23	13
7	6.24	325	08	1.87	0.06	1.93	0.17	0.03	0.20	9
8	6.26	315	08	1.81	0.11	1.92	0.16	0.05	0.21	15
Average	*6.26*	*347*	*08*	*2.22*	*0.06*	*2.29*	*0.17*	*0.03*	*0.20*	*8*

b. Period immediately after *the* first *injection of ricin.*

Date 1906 Jan.	Body weight kilos.	Volume of urine cc.	Sp. gr. of urine 1.0xx.	N of urine grams.	N of feces gram.	Total N grams.	S of urine gram.	S of feces gram.	Total S gram.	Weight of dry feces grams.
9	6.05	480	10	5.18	0.04	5.22	0.34	0.04	0.38	15
10	6.65	340	15	3.66		3.66	0.24		0.24	
11	5.87	410	16	4.42	0.07	4.49	0.29	0.06	0.35	24
12	6.05	185	22	3.67		3.67	0.13		0.13	
13	6.10	280	24	5.55		5.55	0.22		0.22	
14	5.91	353	16	6.94	0.13	7.07	0.28	0.14	0.42	45.5
15	5.71	395	12	4.28	0.08	4.36	0.29	0.09	0.38	29
16	5.67	325	11	3.52	0.05	3.57	0.24	0.06	0.30	19
17	5.67	285	08	3.09	0.05	3.14	0.20	0.06	0.26	19
Average	*5.96*	*339*	*15*	*4.48*	*0.04*	*4.52*	*0.24*	*0.05*	*0.29*	*16*

B. Second Part. High *plane of protein metabolism.*

a. Period immediately before *the* second *injection of ricin.*

Date 1906	Body weight kilos.	Volume of urine cc.	Sp. gr. of urine 1.0xx.	N of urine grams.	N of feces gram.	Total N grams.	S of urine gram.	S of feces gram.	Total S gram.	Weight of dry feces grams.
April										
11	8.57	480	27	11.97	0.83	12.80	0.76	0.15	0.91	38
12	8.56	525	25	13.09	0.90	13.99	0.85	0.17	1.02	41
13	8.63	470	25	11.72	0.84	12.56	0.76	0.15	0.91	38
14	8.63	475	25	12.02	1.16	13.18	0.84	0.21	1.05	53
15	8.67	460	27	11.65	0.78	12.43	0.82	0.14	0.96	35
16	8.69	525	26	13.29	1.22	14.51	0.93	0.22	1.15	56
Average	*8.62*	*489*	*26*	*12.29*	*0.95*	*13.24*	*0.82*	*0.17*	*1.00*	*43*

b. Period immediately AFTER *the* SECOND *injection of ricin.*

DATE 1906 APR.	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.	WEIGHT OF DRY FECES GRAMS.
17	8.43	770	23	19.96	0.36	20.32	1.30	0.07	1.37	17
18	8.43	470	28	12.19	0.35	12.54	0.79	0.06	0.85	17
19	8.58	400	35	10.37	0.92	11.29	0.67	0.17	0.84	44
20	8.64	450	33	12.82	1.24	13.06	0.89	0.23	1.12	59
21	8.65	535	27	15.24	0.66	15.90	1.06	0.13	1.19	31
22	8.69	480	28	13.67	0.87	14.54	0.95	0.17	1.12	41
23	8.68	500	29	13.35	1.22	14.57	0.83	0.23	1.06	58
AVERAGE	*8.58*	*515*	*29*	*13.94*	*0.80*	*14.74*	*0.92*	*0.15*	*1.08*	*38*

equalling 2.11 grams (0.27 gram of N per kilo) and after August 17 she was allowed to run about freely in all the rooms of this large laboratory, no effort being made to collect samples of excreta. During the warm weather of the late summer and early autumn there was little of note in her behavior; there was a constant though gradual fall in weight, but the diet was not notably inadequate until October. Then a more rapid loss in weight was experienced and there was a notable lessening of activity, which became more and more marked. The body weights during this time were not recorded daily. The following data from the records give an accurate idea of the gradual loss until October and the more rapid loss later: August 1, 7.59 k.; August 16, 7.27 k.; September 4, 7.07 k.; October 8, 6.67 k.; November 3, 5.75 k.; December 12, 5.20 k. In December it became apparent that this dog was no longer in health and although the restrictions on the diet were relaxed in January, 1907, she refused to eat, and a diarrhea developed which was fatal (January 25).

SECOND RICIN EXPERIMENT. *Subcutaneous injection of ricin in a dog on a* medium *plane of protein nutrition.* This experiment was designed to show the metabolic effects of ricin on a dog fed with such quantities of food as experience has shown to be ample, but neither excessive nor meager, in protein.

The animal was a mongrel of the hound type, weighing, on September 27, '05, 15.6 kilos. He was not fat, but in good condition on this date. The daily diet consisted of 355 grams of meat, 60 grams of cracker meal, 30 grams of lard, 10 grams of bone ash and 450 cc. of water. The food con-

tained 17.35 grams of nitrogen (1.1 gram per kilo). The weight remained nearly constant; on the date of inoculation, October 16, it was 15.52 kilos. On that date, 15 mgm. of ricin were injected subcutaneously. The following notes were made subsequent to that injection:

October 16, 12 m. Two hours after the inoculation. The dog does not act sick, has no fever.

October 17, 10 a.m. Lies on the floor of the cage; acts sick. Respiratory movements rapid, pulse rapid, nose hot and dry. *4 p.m.* Condition unchanged except that heart action is irregular. There is evidently some irritation at the site of injection.

October 18, 11 a.m. Better today. Moves when coaxed but prefers to lie quietly. Respiratory distress still present but less marked. Nose is cold. Looks stupid and sick. *4 p.m.* Condition unchanged, pulse still irregular in force and rhythm. *5 p.m.* Considerable fever. No other change. Has taken about three-fourths of the day's allowance of mixed food and water.

October 19, 9:30 a.m. Condition much improved, pulse regular, no respiratory distress. There is ulceration and slough at place of injection. A dressing of aluminium acetate applied. *3 p.m.* Dog is asleep and quiet. *4 p.m.* Nauseated but no vomiting. *8 p.m.* Ate balance of food for the day quite eagerly; nose is cold.

On October 20, the condition, aside from the local reaction, appeared almost normal. His convalescence thereafter presented nothing of note. The slough at the site of injection granulated quickly and healed.

The metabolic data obtained for this post-injection period present several features of interest. (See Table II.) The *increased* elimination of nitrogen and of sulphur are approximate measures of the augmented protein catabolism due to the poison. The average daily elimination of nitrogen for the six days preceding the injection was 13.1 grams and for the six days after it, 15.7 grams, the corresponding sulphur elimination being 0.8 gram and 1 gram respectively. There was not the marked increase in the volume of urine following the injection that was noted in some of the other experiments to be described; the daily averages for both periods are practically equal. There was no marked fall in weight, the fluctuations depending largely on the amounts of urine excreted.

THIRD RICIN EXPERIMENT. *Subcutaneous injection of ricin in a dog on a low plane of protein nutrition.*

The dog used in this experiment was a male fox terrier, weighing, on November 10, 1905, 7.05 kilos. He was given daily a diet of 105 grams of meat, 25 grams of cracker meal, 17 grams of lard, 7 grams of bone ash and

TABLE II.

SECOND RICIN EXPERIMENT. Medium *plane of protein metabolism.*
Period immediately BEFORE *the injection of ricin.*

DATE 1905 OCT.	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.	WEIGHT OF DRY FECES GRAMS.
11	15.17	554	29	14.71	0.80	15.51	0.79	0.13	0.92	24
12	15.23	443	27	11.76	0.88	12.64	0.64	0.15	0.79	26
13	15.40	400	24	10.62	0.53	11.15	0.67	0.09	0.66	16
14	15.23	630	24	17.31	0.63	17.94	0.90	0.11	1.01	19
15	15.16	460	27	12.64	0.47	13.10	0.81	0.08	0.89	14
16	15.52	310	37	8.52		8.52	0.54		0.54	
AVERAGE	*15.28*	*466*	*28*	*12.59*	*0.55*	*13.14*	*0.71*	*0.09*	*0.80*	*16*

Period immediately AFTER *the injection of ricin.*

DATE 1905 OCT.	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.	WEIGHT OF DRY FECES GRAMS.
17	15.32	305	39	10.54	0.78	11.32	0.59	0.19	0.78	30
18	15.28	530	37	18.31	0.99	19.30	1.02	0.24	1.26	38
19	15.46	310	35	10.71		10.71	0.59		0.59	
20	15.57	590	29	18.12	0.90	19.02	1.06	0.21	1.27	34
21	15.42	427	26	13.12		13.12	0.77		0.77	
22	15.08	665	30	20.41	0.58	20.99	1.20	0.14	1.34	23.5
AVERAGE	*15.35*	*471*	*32*	*15.20*	*0.54*	*15.76*	*0.86*	*0.13*	*1.00*	*20*

350 cc. of water. The amount of meat in the diet was successively decreased in the daily allowance until it was 69 grams (December 31). Meanwhile the animal's weight fell from 7.05 kilos to 6.1 kilos. On December 31, it was found that the daily average nitrogen elimination for December 29–31, inclusive, was 2.22 grams (urine 1.83 gram; feces 0.39 gram) while the corresponding amount of food nitrogen was 2.83 grams.

No further change was made in the daily quantity of food during January and February (meat 69 grams; other ingredients as above). There was, however, a slow and constant decrease in weight, from 6.12 kilos on January 1, 1906, to 5.63 kilos on February 28. The animal apparently was in perfect health, as he was active and playful. In the following nine days the meat was successively decreased 3 grams daily until 42 grams were the daily allowance. This amount of meat was fed daily during the period following the injection of ricin (2.01 grams of nitrogen or 0.37 gram per kilo).

On March 14, 1906, at 3 p.m., ricin in a dose of 1 mgm. per kilo (5.3 mgm.) was given the dog by hypodermic injection. Records of the observations follow.

March 15, 8 p.m. Dog distressed, not at all lively; marked contrast to usual behavior. *11 p.m.* About same condition, perhaps more reluctant to move. Nose is warmer.

TABLE III.

THIRD RICIN EXPERIMENT. Low *plane of protein metabolism.*

Period immediately BEFORE *the injection of ricin.*

DATE 1906 MAR.	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.	WEIGHT OF DRY FECES GRAM.
9	5.46	330	09	2.53	0.16	2.69	0.25	0.04	0.29	10
10	5.46	325	08	2.49	0.13	2.62	0.25	0.03	0.28	8
11	5.41	345	09	2.64	0.32	2.96	0.27	0.08	0.35	20
12	5.41	335	07	2.40	0.11	2.51	0.26	0.02	0.28	7
13	5.42	305	07	2.18	0.11	2.29	0.23	0.03	0.26	7
14	5.39	307	09	2.19	0.32	2.51	0.24	0.08	0.31	18
AVERAGE	*5.42*	*308*	*08*	*2.40*	*0.19*	*2.59*	*0.25*	*0.04*	*0.29*	*8*

Period immediately AFTER *the injection of ricin.*

15	5.16	463	11	4.14	0.21	4.35	0.15	0.02	0.17	9
16	5.13	215	18	1.36	0.05	1.41	0.13	0.01	0.14	2
AVERAGE	*5.14*	*339*	*14*	*2.75*	*0.13*	*2.88*	*0.14*	*0.01*	*0.15*	*5*

March 16, 9 a.m. Dog looks sick, no respiratory distress, respirations slow. Pulse 120, regular. Temperature, 102.6°. Urine contains trace of albumin. *12 m.* No change. *1 p.m.* Has a chill. *2 p.m.* Begins to show respiratory distress; retraction of abdomen with each inspiration. *4 p.m.* Pulse too rapid to count. Heart rate, 180 (taken with a stethoscope). Temperature 102.2°. Respirations beginning to have peculiar air hunger quality. *5 p.m.* Vomited. *8 p.m.* Has vomited twice since 5 o'clock. Respirations same, heart irregular. *8.45 p.m.* Vomited again. *10.30 p.m.*. Has vomited once since 8.45.

March 17. Died during the night.

Autopsy, March 18. There were no marked pathological lesions other

than minute hemorrhagic areas throughout all the organs. These were more marked in the mesentery than elsewhere. There was a mild grade of fatty degeneration of the heart muscle.

The metabolic data of this experiment, which are presented in Table III, emphasize the conclusions that were drawn in the discussions of the first two experiments.

FOURTH RICIN EXPERIMENT. *Subcutaneous injection of ricin in a dog on a high plane of protein nutrition.*

The subject of this observation was a mongrel male dog weighing, on February 1, 1906, 9 kilos. The food given the animal consisted of 171 grams of meat, 38 grams of cracker meal, 27 grams of lard, 10 grams of bone ash and 450 cc. of water. The meat was gradually increased in amount until, on March 5, the daily allowance was 401 grams. From experience with the other dogs on high protein planes, it did not seem advisable to make any further increase in the protein constituents of the diet and the rapid increase in weight indicated that the allowance was more than ample. During the remainder of the experiment there was no further change in the amounts of the various ingredients of the daily food. The nitrogen in 401 grams of meat and 38 grams of cracker meal, was 14.28 grams (1.4 gram per kilo for 10.03 kilos of body weight on March 1). On this diet there was considerable increase in weight. At the beginning of the experimental period, April 17, the dog weighed 11.09 kilos and was in perfect health in all respects, so far as one could determine. On April 23, 10 mgs. of ricin were administered subcutaneously at 5 p.m. The following notes were subsequently recorded:

April 23, 8 p.m. No noticeable effect. *10 p.m.* Moves only when coaxed, leg evidently very sore. Trembles somewhat. Nose is cold. *11 p.m.* Has vomited. No longer trembles, is bright and active.

April 24, 12.30 a.m. No change since 11 p.m. *9 a.m.* Does not look sick, but refuses to move, evidently due to sore leg. No respiratory symptoms. Temperature 104°. Pulse 90 and regular. *11 a.m.* Refused food and water. *3 p.m.* Dog only touched food, would not eat. Condition unchanged. *8.30 p.m.* No change. *12 p.m.* Ate a very little but did not care for it; wags his tail when spoken to. Appears to be in good condition, except for sore leg.

April 25, 9 a.m. Shows a slight degree of respiratory distress, will not eat; temperature 103.2°. *12 m.* Is very thirsty but will not touch food. Does not look very sick. *4 p.m.* Condition good, walks around cage and appears to be happy. *6 p.m.* No marked change, but seems to be a little better.

April 26. 1 a.m. Condition unchanged. *8 a.m.* Dead.

Autopsy, April 26: Thrombosis of iliac artery due to ricin injection into a small branch. Localized peritonitis, general hemorrhages of all serous surfaces.

The accompanying table (IV) shows the same general effects of ricin on catabolism that were noted to some extent in all these experiments.

TABLE IV.

FOURTH RICIN EXPERIMENT. **High** *plane of protein metabolism. Period immediately* BEFORE *the injection of ricin.*

DATE 1906 APRIL.	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAMS.	S OF FECES GRAMS.	TOTAL S GRAMS.	WEIGHT OF DRY FECES GRAMS.
18	11.14	450	29	12.44	1.08	13.52	0.72	0.27	0.99	62.5
19	11.08	475	27	12.13	0.70	12.83	0.76	0.17	0.93	40
20	11.15	445	29	12.30	0.26	12.56	0.71	0.07	0.78	15
21	11.17	535	25	13.43	0.73	14.16	0.81	0.18	0.99	42
22	11.14	490	27	12.30	0.90	13.20	0.74	0.23	0.97	53
23	11.19	505	26	12.68	0.80	13.48	0.76	0.23	0.99	46
AVERAGE	*11.14*	*500*	*27*	*12.54*	*0.74*	*13.29*	*0.75*	*0.19*	*0.94*	*31*

Period immediately AFTER *the injection of recin.*

DATE 1906 APRIL.	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAMS.	S OF FECES GRAMS.	TOTAL S GRAMS.	WEIGHT OF DRY FECES GRAMS.
24	10.91	660	26	14.40	1.74	16.14	1.07	0.23	1.30	63
25	10.72	290	38	6.33		6.33*	0.47		0.47	
26		155	21	3.38		3.38	0.25		0.25	
AVERAGE	*10.81*	*368*	*28*	*8.03*	*0.58*	*8.61*	*0.59*	*0.07*	*0.67*	*20*

*Did not eat.

FIFTH RICIN EXPERIMENT. *Subcutaneous injection of ricin in a dog on a* **high** *plane of protein nutrition.* The object of this experiment was to test the resistance of a dog on a *particularly* high plane of protein nutrition.

The animal was a well nourished, male fox terrier. At the beginning of the experiment, on November 22, 1905, the dog weighed 10.58 kilos. He was put on the usual diet, which consisted of 150 grams of meat, 45 grams of cracker meal, 25 grams of lard and 12 grams of bone ash, with 500 cc. of water. The amount of meat was increased during December, at first by 20 gram daily increments, later by 50 gram additions, so that on December 31 the daily meat allowance was 1160 grams. The total amount of nitrogen in the daily food was 42.53 grams (3.37 grams of N per kilo).

Meanwhile the dog increased rapidly in weight—on January 1, it was 12.59 kilos. On January 2, 4, 5 and 6, there was some vomiting at various intervals after feeding and on January 7, he refused to eat. There was occasional vomiting for several days, also some diarrhea, and in consequence only water was given on January 10 and 11. On the 14th feeding was resumed with 150 grams of meat in the diet and continued in this amount through the experimental period (N of food was 6.12 grams; 0.51 gram per kilo on 12 kilo basis).

The body weight of the dog on the day of administering the toxin was 13.03 kilos and 13.4 mgm. of ricin were given under the usual conditions. These notes were recorded:

Januray 28, 5:50 p.m., six hours after injection. The dog has vomited and there have been diarrheal discharges. Is panting and feverish, but does not seem uncomfortable; is quite lively. Temperature 105.6°. *6:45 p.m.* Resting quietly. Not at all sick looking; nose is cold. *8:45 p.m.* Same condition.

January 29, 12:15 a.m. Condition not changed. *8:30 a.m.* Bright; wags his tail when spoken to, but refuses to move (pain in the leg due to inoculation). No respiratory distress. Temperature 104°. *11 a.m.* Drank water from food mixture but refused to eat. *1 p.m.* Ate quite eagerly about two-thirds of food mixture. Temperature 103.3°. *10:30 p.m.* Moves about cage when coaxed; not animated but does not act sluggish nor sick. Refused to eat remainder of food.

January 30, 10 a.m. More apathetic than yesterday. Respirations more rapid and labored. Pulse regular. *11. a.m.* Temperature 102.6°. Refused to eat or drink. *3 p.m.* Temperature 100.8°. Is much more sick than he was this morning. *9:30 p.m.* Dead. See Table V.

Autopsy. January 31, Rigor mortis present. Wide area of induration about site of inoculation. The subcutaneous tissue and fat are very edematous on left side to within 3 in. of costal margin. The muscle at site of inoculation is dark red, boggy and shows ecchymosis. The artery and vein (iliacs) are clear and without sign of thrombi. Abdominal cavity contain four drams of dark sanguinous fluid. The omentum and mesentery are intensely congested and show areas of hemorrhage. The intestinal vessels are engorged. Liver and spleen appear normal, kidneys are normal. The intestine on being opened shows intense congestion from duodenum to rectum. From the ileocecal valve down there is bloody mucus on the mucosa which is covered with ecchymoses. Stomach and gall bladder appear normal. *Heart:* Normal in size. On epicardium, along course of coronaries, are ecchymotic areas from pin point to pin head size; coronaries show no thrombi. Heart muscle is dark in color but shows no sign of hemorrhages or fatty degeneration. *Lungs:* Left lung is darker in color than right, more resistant to touch and less crepitant. On cuts surfaces appear numerous areas of hemorrhage 1 mm. in diameter; tissue floats (hypostatic congestion). Right lung seems normal.

SIXTH RICIN EXPERIMENT. *Subcutaneous injection of ricin in a dog on a* **high** *plane of protein nutrition.*

TABLE V.

FIFTH RICIN EXPERIMENT. **High** *plane of protein metabolism. Period immediately* BEFORE *the injection of ricin.*

DATE 1906 JAN.	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.	WEIGHT OF DRY FECES GRAM.
23	12.98	620	27	16.22	1.11	17.33	0.93	0.27	1.20	58.5
24	13.05	600	25	15.70	0.52	16.22	0.90	0.14	1.04	27.5
25	13.03	683	21	17.87	1.20	19.07	1.02	0.31	1.33	63
26	13.17	518	27	14.10	0.52	14.62	0.93	0.13	0.96	27
27	13.17	630	29	17.11	0.34	17.45	1.13	0.09	1.22	18
28	13.42	670	27	18.45	0.24	18.69	1.20	0.06	1.26	13
AVERAGE	*13.13*	*620*	*26*	*16.57*	*0.65*	*17.23*	*1.00*	*0.16*	*1.16*	*34*

Period immediately AFTER *the injection of ricin.*

DATE	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.	WEIGHT OF DRY FECES GRAM.
29	12.95	630*	29	25.51*	1.63*	26.14	1.13	0.51	1.64	100*
30	12.79	250*	34	10.12	0.49	10.63	0.74	0.15	0.89	30
AVERAGE	*12.87*	*440*	*31*	*17.81*	*1.06*	*18.38*	*0.93*	*0.33*	*1.26*	*65*

*Both urine and feces contained slight amounts of vomitus.

The dog used in this experiment was a fox terrier weighing 8.9 kilos on April 9, 1906, when the regular feeding was commenced. The diet consisted, at the beginning, of 135 grams of meat, 40 grams of cracker meal, 27 grams of lard, 9 grams of bone ash and 360 cc. of water. The meat was cumulatively increased a few grams daily until the allowance was 400 grams (June 4), at which figure it was held during the remainder of the period. This diet contained 15.08 grams of nitrogen per day (or about 1.5 gram per kilo). At the beginning of the preliminary period, July 3, the dog's weight had increased to 10.15 kilos. On July 8, 10 mgs. of ricin were injected at 8 pm. The following notes give the data that were subsequently recorded.

July 9, 10 a.m. Ate food as usual. *11 a.m.* Temperature 103°. *12 m.* Vomited. *2 p.m.* Apparently becoming more quiet. *4 p.m.* erhaps somewhat worse; pronounced dyspnœa. Heart action weak.

TABLE VII.

A summary of Average *metabolic data, per period, for the* RICIN *experiments* (1-6)

FIRST EXPERIMENT. FIRST PART. Low *plane of protein metabolism.*

	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.
Before injection	6.26	347	2.22	0.06	2.29	0.17	0.03	0.20
After injection	5.96	339	4.48	0.04	4.52	0.24	0.05	0.29

FIRST EXPERIMENT. SECOND PART. High *plane of protein metabolism.*

Before injection	8.62	489	12.29	0.95	13.24	0.82	0.17	1.00
After injection	8.58	515	13.94	0.80	14.74	0.92	0.15	1.08

SECOND EXPERIMENT. Medium *plane of protein metabolism.*

Before injection	15.28	466	12.59	0.55	13.14	0.71	0.09	0.80
After injection	15.35	471	15.20	0.54	15.76	0.86	0.13	1.00

THIRD EXPERIMENT. Low *plane of protein metabolism.*

Before injection	5.42	308	2.40	0.19	2.59	0.25	0.04	0.29
After injection	5.14	339	2.75	0.13	2.88	0.14	0.01	0.15

FOURTH EXPERIMENT. High *plane of protein metabolism.*

Before injection	11.14	500	12.54	0.74	13.29	0.75	0.19	0.94
After injection	10.81	368	8.03	0.58	8.61	0.59	0.07	0.67

FIFTH EXPERIMENT. High *plane of protein metabolism.*

Before injection	13.13	620	16.57	0.65	17.23	1.00	0.16	1.16
After injection	12.87	440	17.81	1.06	18.38	0.93	0.33	1.26

SIXTH EXPERIMENT. High *plane of protein metabolism.*

Before injection	10.21	362	10.63	0.95	11.58	0.65	0.28	0.93
After injection	9.96	280	7.81	0.40	8.22	0.51	0.12	0.63

July 13, 9 a.m. Worse again this morning. Site of injection looks vicious, drank 135 cc. of water. *1 p.m.* No change in condition. Drank 60 cc. of water. *8 p.m.* Dog very restless, leg pains him; slough size of a silver dollar separating. Temperature 103°. *12 p.m.* Rapidly growing worse.

July 14, 8 a.m. Dead.

Autopsy, July 14. Same lesions as those noted in other cases.

The metabolic data of this experiment are recorded, in part in Table VI. See also Table VII.

HEMORRHAGE EXPERIMENTS.

It was planned to make the second section of this research a study of the effects of hemorrhage. It was believed that severe though non-fatal hemorrhage tests the stamina of animals as fully as any other imposed condition because of the immediate effects of the blood removal ("shock") and the subsequent continuous processes of blood renewal. I am quite aware that the effects of hemorrhage on animals cannot at present be interpreted as indicating any degree of immunity to disease processes which arise from bacterial infections, although it seems probable that there is some relation between immunity and the power of blood renewal. However, I think that inasmuch as blood must be considered a tissue, the ability of an animal to withstand severe and repeated hemorrhages gives some indication of that animal's recuperative powers and a measure of the reserves on which the organism calls for tissue repair. This phase of the investigation is moreover very practical, for while modern surgery is almost bloodless, accidents causing severe losses of blood are more common now than formerly.

It has been shown in this laboratory, by Hawk and Gies,[1] that external hemorrhage causes increased metabolism, as evidenced by increased output of nitrogen in the urine. It follows that if dogs on low planes of protein nutrition do not have the surplus protein required for the formation of new blood and the production of the inevitably attendant waste products, they will not be able to withstand the mechanical loss of a large quantity of blood or even the frequent losses of small quantities.

The plan of these hemorrhage experiments, similar to that of the ricin experiments, was to give dogs definite quantities of food

[1] Hawk and Gies: *Amer. Journ. of Physiol.*, xi, p. 171, 1904.

daily for considerable periods of time, thus bringing the animals to certain nutritional planes, then to withdraw from a femoral artery an amount of blood equivalent to a certain percentage of the body weight, and subsequently to determine comparative metabolic features. A series of three bleedings was usually carried out in each experiment, as will be explained below.

It has been ascertained by experiments in this laboratory that ordinarily well fed healthy dogs are capable of losing 3 per cent of their body weight in blood without any particular risk of life but a loss of 3.5 per cent is usually not devoid of danger. If, however, so much blood as 4 per cent of the body weight is withdrawn from a femoral artery,[1] the operation must be conducted slowly and cautiously, for the effect on the animal is very marked and death not infrequent.

In these experiments I undertook at the time of the first of each series of hemorrhages from a dog, to approximate 4 per cent of body weight in the amount of blood taken, but in some cases the condition of the animal, as evidenced by the heart action and especially by the respiratory movements, indicated that a danger point had been passed before the amount of blood desired had been obtained.[2]

The second bleeding of each dog was performed eight days after the first[3] and four days later, if the animal survived, it was bled a third time.

First hemorrhage experiment. *Hemorrhage from a femoral artery of a dog on a low plane of protein nutrition.*

The first dog selected for this hemorrhage work was a fox terrier mongrel, weighing 9 kilos. The animal was put on a diet of 80 grams of meat, 32 grams of cracker meal and 25 grams of lard with 8 grams of bone ash. The total allowance of water was 280 cc. The meat portion of the diet was slowly decreased daily between October 24, 1906 (the day on which the experiment was begun) and January 26, 1907. By the latter date,

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[1] Not infrequently the blood ceases to flow before 4 per cent of the body weight has been withdrawn, even when no clots are discernible in the blood vessel or cannula.

[2] In dogs that are being bled spasmodic contraction of the neck muscles (dyspnoea), is manifested when danger is imminent.

[3] Exceptions are noted in the descriptions of experiments I, V and VI.

the dog was ingesting daily 64 grams of meat. The daily diet then contained 3.39 grams of nitrogen (0.44 gram of N *per kilo*). This diet was continued throughout the remainder of the experiment. The dog's weight during this time had fallen from 9 kilos to 7.73 kilos. Aside from the loss of weight, which was somewhat apparent in the appearance of the dog, the animal seemed to be perfectly normal and was quite active. The first hemorrhage was conducted on February 12 at which time the dog weighed 7.6 kilos.

February 12. Dog weighs 7.60 k. Anésthetized with ether at 2 p.m. and bled from a femoral artery (about 250 grams). At this point the dog showed signs of respiratory distress, i.e., gasping and convulsive movements of larynx, and it was believed that withdrawal of more blood would produce a fatal result. The bleeding consequently was discontinued. Amount of blood withdrawn: 257 grams, or 3.38 per cent of body weight. Period of withdrawal, 11 minutes. The dog took ether slowly, requiring considerable, and recovered slowly from the anesthetic. The immediate results from the hemorrhage were not marked.

February 13. Dog is rather apathetic but otherwise appears to be normal. Recovery henceforth uneventful.

Table VIII shows some of the metabolic effects of the first hemorrhage.

February 20. Eight days after first bleeding. The dog was again bled from a femoral artery at 3 p.m. The bleeding was continued until the critical signs observed at the first operation again appeared. 182 grams of blood were withdrawn (2.53 percent of the body weight). The same signs of respiratory distress and poor heart action appeared. The dog required little ether and recovered from the anesthetic about the time the last blood was withdrawn. After the operation the animal was returned to a warm cage. He rallied feebly and appeared to be recovering satisfactorily on the night of the operation but died during the night.

SECOND HEMORRHAGE EXPERIMENT. *Hemorrhage from a femoral artery of a dog on a high plane of protein nutrition.*

The dog in this experiment was a mongrel Irish terrier, weighing 5.8 kilos. On November 20, 1906, he was put on a daily diet of 90 grams of meat, 25 grams of cracker meal, 18 grams of lard, with 6 grams of bone ash and 240 cc. of water. This diet was cumulatively increased daily in its meat constituent until December 30, when the animal received 200 grams of meat. The daily diet was held at this amount until the time of the first hemorrhage, on February 19, 1907. The total amount of nitrogen ingested daily after December 30 was 9.43 grams, or 1.57 gram per kilo. The weight of the animal increased from 5.8 kilos to 6 kilos on the day before the first hemorrhage.

TABLE VIII.

FIRST HEMORRHAGE EXPERIMENT. Low *plane of protein metabolism.*
Period immediately BEFORE *hemorrhage.*

DATE 1907 FEB.	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.	WEIGHT OF DRY FECES GRAM.
7	7.65	280	11	2.58	0.20	2.78	0.17	0.05	0.22	13
8	7.67	240	12	2.21	0.20	2.41	0.14	0.05	0.20	13
9	7.63	275	11	2.53	0.34	2.88	0.16	0.09	0.26	23
10	7.65	240	14	2.24	0.34	2.58	0.14	0.09	0.23	23
11	7.64	300	10	2.80	0.22	3.02	0.17	0.06	0.24	15
12	7.60	290	13	2.70	0.17	2.87	0.17	0.04	0.21	11
AVERAGE	7.64	270	12	2.51	0.24	2.76	0.16	0.06	0.23	14
Period immediately AFTER *hemorrhage (3.38% of body weight.)*										
13	7.35	160	21	2.79	0.18	2.97	0.16	0.05	0.20	13
14	7.32	225	20	3.93	0.25	4.17	0.22	0.06	0.28	18
15	7.25	280	18	4.88	0.21	5.09	0.28	0.05	0.33	15
16	7.29	200	17	3.06	0.33	3.39	0.23	0.08	0.32	24
17	7.27	250	18	3.82	0.46	4.27	0.29	0.12	0.41	33
18	7.26	225	25	3.44	0.43	3.87	0.26	0.11	0.37	32
AVERAGE	7.29	223	20	3.65	0.31	3.96	0.24	0.08	0.32	22

First hemorrhage. February 19. The dog weighs exactly 6 kilos. The anesthetic (ether) was well taken at 2 p.m. and very little was required to produce complete anesthesia. Bleeding was done from a femoral artery as usual and 259 grams of blood withdrawn, 4.3 per cent of the animal's weight. There was no indication at any time that the dog was in a dangerous condition. Period of bleeding, 12 minutes. The dog recovered from the effects of the ether almost immediately and in 30 minutes was walking about the cage. In two hours he was active and alert. and showed none of the usual signs of exhaustion following hemorrhage.

February 20. The dog is almost normal. Runs about cage and seems happy but is not quite so lively as usual. In other respects apparently normal.

February 21. So far as may be observed the dog is quite normal.

Table IX gives the recorded analytic and metabolic data.

Second hemorrhage. February 24. It was intended to bleed this animal again on this date but during the night she pulled the stitches from the

old incision and bled copiously therefrom. The day's urine contained a large blood clot. The total amount of urine and blood was 475 cc. Subtracting from this total the average amount of urine excreted (275 cc.) there is a remainder of 200 cc. As the clot in the urine, after it had been freed of as much urine and serum as possible, weighed 265 grams, it is safe to say that at least 250 grams of blood were lost by the animal. Probably the amount was greater than this, since doubtless the dog lapped up all she could of it during the hemorrhage. Estimating the percentage lost on the minimum basis (250 grams) the animal in this accidental hemorrhage eliminated 4.3 per cent of her weight. At 10 a.m., she acts perfectly normal, and is active and very restless while her food is being prepared for her (increased appetite).

February 25. So far as one can observe the dog is quite normal. (See table IX).

Third hemorrhage. February 28. The dog now weighs 5.8 kilos and is in excellent condition. She was anesthetized about two o'clock and bled from the right femoral. The intention was to exsanguinate the animal to the point of danger. When 180 grams of blood had been withdrawn, no more could be taken as it would not run, no clot or obstruction being discernible. The animal began to gasp and show contraction of laryngeal muscles. The wound was immediately closed and the dog replaced in a warm cage. Twenty minutes after the operation the dog staggered weakly around the cage but had no respiratory distress. The heart action was strong though rapid. The amount of blood taken was equal to 3.1 per cen tof the body weight. Period of withdrawal, 13 minutes.

March 1. The dog is more quiet than usual but in other respects does not act abnormally. She eats her food eagerly.

March 2. The manner of the animal is quite normal.

From this date forward there was nothing in the condition of this dog to excite notice. She appeared entirely normal in every respect. The dog was subsequently under observation for about ten days. (See table IX).

THIRD HEMORRHAGE EXPERIMENT. *Hemorrhage from a femoral artery of a dog on a high plane of protein nutrition.*

The animal used in this experiment was of a collie type, male, weighing, on February 23, 1907, 11.2 kilos. He was put on the following diet: 165 grams of meat, 44 grams of cracker meal, 33 grams of lard, with 15 grams of bone ash and 285 cc. of water. The meat of the diet was cumulatively increased daily (the other ingredients remaining the same) until, by March 15, the dog was receiving 500 grams of meat per day. The total nitrogen of the food under these conditions was 18.71 grams daily (meat, 18.05 grams; cracker meal, 0.66 gram). The animal slowly gained in weight until the beginning of the preliminary period, April 27, when he weighed 12.65 kilos. The weight, as will be observed

TABLE IX.

SECOND HEMORRHAGE EXPERIMENT. High *plane of protein metabolism.*
FIRST PERIOD. *Immediately* BEFORE *hemorrhage.*

DATE 1907 FEB.	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.	WEIGHT OF DRY FECES GRAMS.
14	6.00	240	25	4.77	0.35	5.11	0.31	0.09	0.40	32
15	6.03	265	24	5.26	0.34	5.60	0.34	0.08	0.42	31
16	6.02	290	24	5.76	0.67	6.43	0.38	0.15	0.53	62
17	6.00	325	21	7.02	0.13	7.15	0.42	0.03	0.45	12
18	5.95	335	22	7.24	0.63	7.87	0.43	0.14	0.57	58
19	6.00	290	23	6.27	0.12	6.39	0.38	0.03	0.41	11
AVERAGE	*6.00*	*290*	*23*	*6.05*	*0.37*	*6.42*	*0.37*	*0.09*	*0.46*	*34*

SECOND PERIOD. AFTER *the* FIRST *hemorrhage (4.31 per cent of body weight).*

20	5.75	235	31	5.84	0.52	6.36	0.35	0.13	0.48	45
21	5.73	295	21	7.33	0.44	7.77	0.44	0.11	0.55	38
22	5.78	295	25	7.33	0.33	7.67	0.44	0.08	0.52	29
23	5.79	275	23	6.23	0.38	6.61	0.41	0.09	0.50	36
AVERAGE	*5.76*	*275*	*25*	*6.68*	*0.42*	*7.10*	*0.41*	*0.10*	*0.51*	*37*

THIRD PERIOD. AFTER *the* SECOND *hemorrhage (4.3 per cent of body weight).*

24	5.68	180	27	3.80	0.13	3.93	0.25	0.03	0.28	12
25	5.70	295	22	7.67	0.41	8.09	0.50	0.09	0.60	36
26	5.74	280	23	7.28	0.24	7.52	0.47	0.05	0.53	21
27	5.77	200	33	5.20	0.32	5.52	0.34	0.07	0.41	28
28	5.80	280	26	6.59	0.40	6.99	0.39	0.08	0.48	35
AVERAGE	*5.76*	*247*	*26*	*6.10*	*0.30*	*6.41*	*0.39*	*0.06*	*0.46*	*26*

FOURTH PERIOD. AFTER *the* THIRD *hemorrhage (3.1 per cent of body weight).*

Mar.										
1	5.67	270	26	6.35	0.18	6.54	0.38	0.04	0.43	16
2	5.70	290	22	6.98	0.34	7.33	0.41	0.07	0.49	30
3	5.69	315	20	7.05	0.41	7.47	0.45	0.09	0.55	36
4	5.71	305	21	6.82	0.28	7.10	0.44	0.06	0.50	24
5	5.73	265	25	5.93	0.31	6.24	0.37	0.06	0.44	27
AVERAGE	*5.70*	*289*	*23*	*6.62*	*0.30*	*6.93*	*0.41*	*0.06*	*0.48*	*26*

from the records in table X, continued to increase slowly. Taking the average weight for the preliminary period as 12.8, the animal at that time ingested daily about 1.4 gram of nitrogen per kilo.

First hemorrhage. The first hemorrhage was made on May 2, 1907, at 4 o'clock—6 hours after feeding. The dog took the ether badly, considerable anesthetic being required and his breathing was peculiar throughout the operation, A femoral artery was exposed, and a cannula was inserted with some difficulty because the artery was unusually small for a dog of this size. About 490 cc. of blood were slowly withdrawn. The period of withdrawal was 11 minutes. Bleeding was stopped at this point because the dog appeared to be in a precarious condition. He recovered from the anesthetic in half an hour but was too weak, apparently, to move about the cage. He did not appear particularly sick, wagged his tail when spoken to, and raised his head. The amount of blood withdrawn was 3.8 per cent of the animal's weight.

May 3. The dog vomited during the night and also had violent diarrheal discharges containing blood and mucus. He was now very apathetic, and would not move even when coaxed but lay quietly in the cage. Showed no interest in anything. Refused to eat his food when it was offered to him but drank 385 cc. of water. His temperature was normal.

May 4. The dog is still very logy. He will not move except when compelled to do so. Ate only half of his food today and this apparently without any relish. His temperature is normal.

May 5. The dog is still very quiet, has not been observed to move about the cage voluntarily. Careful examination of the animal was made but nothing unsatisfactory in his general condition could be detected. The wound is clean and healing satisfactorily. There is no fever.

May 6. The dog looks bright today; he is not active, shows no inclination to move about but lies quietly in the cage. He ate his food after urging.

May 8. Same condition.

May 9. Today is the first day that the dog has acted normally. (See Table X.)

Second hemorrhage. May 10. 3 p.m. The dog was again put under anesthesia, which he took without difficulty. The femoral artery of the other leg was exposed and 294 grams of blood were withdrawn. (2.3 per cent of the body weight). The same condition of the artery was observed in this leg as at the time of the first operation, the artery being smaller than normal for a dog of his size. The duration of the hemorrhage was 22 minutes. The blood flowed very slowly, the pressure being quite low. Recovery from the anesthetic was rapid but the animal appeared much more prostrated than the dog in the preceding experiment after the second hemorrhage, although the relative loss of blood was much less than in the case of that animal.

May 11. The dog lies quietly in his cage, wags his tail when he is spoken to, but declines to be coaxed into moving Refuses to eat.

May 12. Same condition.

May 13. The dog is still very listless, will not move voluntarily about the cage, appears to sleep a great deal of the time. (See Table X.)

Third hemorrhage. May 14. Anesthetized at 3 p m. Artery exposed from the site of the first operation up to the pelvis. Artery was completely thrombosed and has assumed the appearance of a nerve. On account of the impossibility of effecting satisfactory hemorrhage from this artery, blood was withdrawn from a femoral vein, but only 85 grams could be secured (0.7 per cent of the body weight). The period of withdrawal of the blood was 15 minutes. The dog was under anesthesia one-half hour. He recovered from the anesthesia very slowly and his condition was apparently the same as that following the other hemorrhages.

May 15. The dog has not eaten. Will not move. Slept most of the day.

May 16. Refuses to eat. Was given 385 cc. water. Temperature is normal.

May 17. Condition unchanged. Careful examination fails to reveal anything as a cause for the apathy in the dog. The wounds from both operations are in a satisfactory condition.

The dog's condition did not improve. His torpor increased and death ensued on the twenty-first of May. (See Table X.)

My impression is that this dog was abnormal to begin with, although, aside from the peculiar arterial condition observed, in both femoral arteries, nothing organically unusual could be discerned. The normal temperature and a normal leucocyte count excluded a septic condition. Autopsy failed to disclose anything to account for the symptoms described.

FOURTH HEMORRHAGE EXPERIMENT. *Hemorrhage from a femoral artery of a dog on a* low *plane of protein nutrition*

The dog selected for this experiment was a "wire" hair, fox terrier, bitch, weighing at the beginning of the experiment, on March 27, 1907, 7.2 kilos. The daily diet at the start consisted of 84 grams of meat, 28 grams of cracker meal, 21 grams of lard, with 7 grams of bone ash, and 245 cc. of water. The meat of the diet was diminished in amount daily until 50 grams remained in the diet on April 16. This was the amount of meat given daily during the rest of the experiment. The amount of nitrogen in the daily food under these conditions was, on April 16, 2.3 grams (0.35 gram per kilo). The dog slowly decreased in weight from 7.2 kilos on March 27 to 6.14 kilos on April 27. The general health of the animal, however, appeared excellent. She was playful, noisy and very good natured.

First hemorrhage. The first bleeding was done on May 2 at 4 p.m. After being anesthetized, a femoral artery was exposed and 262 grams of blood were slowly withdrawn. The period of the hemorrhage was 11

TABLE X.

THIRD HEMORRHAGE EXPERIMENT. High *plane of protein metabolism.*
FIRST PERIOD. *Immediately* BEFORE *hemorrhage.*

Date 1907 Apr.	Body weight kilos.	Volume of urine cc.	Sp. gr. of urine 1.0XX.	N of urine gram.	N of feces gram.	Total N grams.	S of urine gram.	S of feces gram.	Total S gram.	Weight of dry feces grams.
28	12.79	350	44	14.52	0.50	15.02	0.95	0.14	1.09	37
29	12.81	395	37	16.39	0.93	17.32	1.07	0.26	1.33	69
30	12.87	350	41	14.52	0.71	15.23	0.95	0.20	1.15	52
May										
1	12.86	285	41	11.03	0.15	11.18	0.77	0.04	0.81	11
2	12.86	220	42	8.51	0.84	9.35	0.55	0.24	0.79	62
AVERAGE	*12.83*	*300*	*41*	*12.99*	*0.62*	*13.62*	*0.85*	*0.18*	*1.03*	*96*

SECOND PERIOD. AFTER *the* FIRST *hemorrhage (3.8 per cent of body weight).*

Date 1907 May	Body weight kilos.	Volume of urine cc.	Sp. gr. of urine 1.0XX.	N of urine gram.	N of feces gram.	Total N grams.	S of urine gram.	S of feces gram.	Total S gram.	Weight of dry feces grams.
3	11.82	170*	46	6.64†	0.51	7.15	0.34	0.16	0.50	38
4	11.77	205	46	8.01		8.01	0.54		0.54	
5	11.90	235	48	14.26		14.26	0.61		0.61	
6	12.27	365	44	15.88	0.99	16.87	1.02	0.31	1.33	74
7	12.11	495	36	21.53	1.08	22.61	1.38	0.34	1.72	80
8	12.33	240	44	10.44	0.57	11.01	0.67	0.18	0.85	42
9	12.40	360	43	15.93	0.65	16.58	0.98	0.20	1.18	48
10	12.51	330	42	14.80	0.73	15.53	0.90	0.23	1.13	54
AVERAGE	*12.14*	*300*	*43*	*13.43*	*0.56*	*14.00*	*0.80*	*0.23*	*0.98*	*56*

THIRD PERIOD. AFTER *the* SECOND *hemorrhage (2.3 per cent of body weight).*

Date 1907 May	Body weight kilos.	Volume of urine cc.	Sp. gr. of urine 1.0XX.	N of urine gram.	N of feces gram.	Total N grams.	S of urine gram.	S of feces gram.	Total S gram.	Weight of dry feces grams.
11	12.33	190	50	8.88†	0.63	9.51	0.67	0.19	0.86	47
12	11.69	270	50	12.62	0.31	12.93	0.95	0.10	1.05	23
13	12.00	200	57	10.69	0.81	11.50	0.64	0.25	0.89	60
14	12.29	210	54	11.22	0.72	11.94	0.68	0.20	0.88	48
AVERAGE	*12.07*	*217*	*53*	*10.85*	*0.61*	*11.47*	*0.73*	*0.18*	*0.92*	*44*

FOURTH PERIOD. AFTER *the* THIRD *hemorrhage (0.7 per cent of body weight).*

Date 1907 May	Body weight kilos.	Volume of urine cc.	Sp. gr. of urine 1.0XX.	N of urine gram.	N of feces gram.	Total N grams.	S of urine gram.	S of feces gram.	Total S gram.	Weight of dry feces grams.
15	12.02	255	58	12.73†	0.45	13.18	0.74	0.13	0.87	30
16	11.60	150	60	7.49	0.39	7.88	0.43	0.11	0.54	26
17	11.47	155	34	6.52	0.37	6.89	0.43	0.10	0.53	25
18	11.82	250	45	10.87	0.29	11.16	0.64	0.09	0.73	23
19	12.00	305	43	13.27	0.96	14.23	0.75	0.27	1.02	64
AVERAGE	*11.78*	*223*	*48*	*10.18*	*0.49*	*10.67*	*0.60*	*0.14*	*0.74*	*33*

*Small amount of vomitus in urine. †Refused to eat. 355cc. of water given.

minutes. The amount of blood, reckoned in percentage of the body weight, was 4.02 per cent. The animal recovered quickly from the effects of the anesthetic and in half an hour was attentive when spoken to.

May 3. The dog does not act sick in any respect, walked about the cage, is playful and with the exception of being somewhat less restless than normal appears to be in her usual condition.

May 4, The dog is still somewhat more quiet than normal.

May 5. So far as may be seen the animal is quite normal. (See Table XI).

Second hemorrhage. May 10. Under anesthesia at about 2 p.m. The opposite femoral artery was exposed, cannula inserted and 236 grams of blood slowly withdrawn. There were no marked signs of impending danger at any time during the hemorrhage, which lasted 8 minutes. The animal recovered from the anesthetic in 15 minutes, and walked around the cage. In half an hour she was sleeping quietly. The amount of blood taken was equal to 4 per cent of the body weight.

May 11. The dog does not seem to be as much affected by the hemorrhage of yesterday as she was the day following the first hemorrhage. It is quite difficult today to detect anything abnormal in her appearance or activities. (See Table XI.)

Third hemorrhage. May 14. At about 2:30 p.m. a femoral artery was again exposed and 227 grams of blood withdrawn in 10 minutes. There were no signs of danger during the period of the hemorrhage. The arterial pressure was notably strong even at the withdrawal of the last few cubic centimeters. It seemed possible to take more blood without endangering the life of the animal. One-half hour after the operation the dog had entirely recovered from the anesthetic and walked about the cage, but was quite weak. The amount of blood taken was 3.9 per cent of the body weight.

May 15. The animal looks slightly apathetic this morning and is sleeping more than she normally does at this time of the day. She ate her food greedily, as she has done each day during the progress of the experiment.

May 16. The dog is somewhat more quiet than normally but in all other respects there is nothing to be observed that is abnormal. She is still restless, moves about the cage and is willing to play when opportunity is given.

May 19. Recovery uneventful. Seems as well as ever. (See Table XI.)

FIFTH AND SIXTH HEMORRHAGE EXPERIMENTS. *Hemorrhage from femoral artery. Twin dogs on* **high** *and* **low** *planes of protein nutrition.* On account of the disagreements in the resu'ts of the first four hemorrhage experiments it was decided to eliminate, so far as possible, these factors due to inherent differences among the animals, such as breed, etc., by using two animals of the same litter.

TABLE XI

FOURTH HEMORRHAGE EXPERIMENT. Low *plane of protein metabolism*
FIRST PERIOD. *Immediately* BEFORE *hemorrhage.*

DATE 1907 APR.	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.	WEIGHT OF DRY FECES GRAMS.
28	6.15	260	21	2.51	0.20	2.71	0.12	0.05	0.17	14
29	6.11	245	12	2.37	0.34	2.71	0.11	0.08	0.19	23
30	6.11	265	11	2.56	0.22	2.78	0.12	0.05	0.17	15
May										
1	6.10	220	12	2.16	0.22	2.38	0.15	0.05	0.20	15
2	6.10	255	12	2.50	0.06	2.56	0.17	0.01	0.18	4
AVERAGE	*6.11*	*249*	*13*	*2.42*	*0.20*	*2.62*	*0.13*	*0.05*	*0.18*	*14*
SECOND PERIOD. AFTER *the* FIRST *hemorrhage (4.02 per cent of body weight).*										
3	5.88	165	07	2.16	0.04	2.20	0.13	0.01	0.14	3
4	5.88	245	19	3.20	0.06	3.26	0.19	0.02	0.21	5
5	5.86	235	16	3.07	0.22	3.29	0.18	0.08	0.26	17
6	5.85	200	11	1.82	0.21	2.03	0.16	0.07	0.23	16
7	5.89	225	14	2.14	0.13	2.27	0.18	0.05	0.23	10
8	5.97	180	09	1.64	0.17	1.81	0.14	0.06	0.20	13
9	5.95	225	10	2.30	0.30	2.60	0.17	0.11	0.18	23
10	5.90	270	14	2.75	0.22	2.97	0.21	0.08	0.29	17
AVERAGE	*5.89*	*218*	*12*	*2.38*	*0.17*	*2.55*	*0.17*	*0.06*	*0.23*	*13*
THIRD PERIOD. AFTER *the* SECOND *hemorrhage (4 per cent of body weight).*										
11	5.56	325	10	3.01	0.14	3.15	0.18	0.05	0.23	9
12	5.63	155	18	1.44	0.32	1.76	0.09	0.11	0.20	21
13	5.68	185	14	2.16	0.15	2.31	0.14	0.05	0.19	10
14	5.69	230	15	3.39	0.11	3.50	0.17	0.04	0.21	7
AVERAGE	*5.64*	*224*	*14*	*2.50*	*0.18*	*2.68*	*0.14*	*0.06*	*0.20*	*12*
FOURTH PERIOD. AFTER *the* THIRD *hemorrhage (3.9 per cent of body weight).*										
15	5.40	225	14	2.94	0.33	3.27	0.18	0.12	0.30	22
16	5.50	150	23	2.03	0.36	2.39	0.12	0.13	0.25	24
17	5.49	235	13	2.77	0.26	3.03	0.19	0.09	0.28	17
18	5.50	210	12	2.48	0.26	2.74	0.17	0.09	0.26	17
19	5.51	215	15	2.54	0.15	2.69	0.17	0.05	0.22	10
AVERAGE	*5.48*	*207*	*15*	*2.55*	*0.27*	*2.82*	*0.16*	*0.09*	*0.26*	*18*

Of course it was not imagined that two such dogs would be *identical* in their physiological resistance, but it was taken for granted that two such animals would be as much alike as two animals not thus related could be. The dogs used in the next two experiments were Scotch terriers about two years of age, a male and a female, which, since their birth, had been together and lived under identical conditions. The male was slightly heavier, weighing 9 kilos; the female about 8.5 kilos. The female was somewhat more active, and gave one the general impression of having slightly more endurance. On that account she was put upon a low nitrogen plane of nutrition; her brother was put upon a high plane. (See Tables XII and XIII).

FIFTH HEMORRHAGE EXPERIMENT. *Twin dog on a low plane of protein nutrition. (Compare with the sixth hemorrhage experiment).*

On April 9, 1908, the female Scotch terrier was put upon the following diet: 100 grams of meat, 33 grams of cracker meal, 25 grams of lard, with 10 grams of bone ash and 315 cc. of water. With this diet, between April 9 and 13, the dog maintained practically a constant weight of 8.47 kilos. The amount of meat was reduced 10 grams on April 13th, and again 10 grams on the 15th and another 10 grams on the 17th, making on the 17th of April a diet of 70 grams of meat. On April 22 the dog's weight was 8.39 kilos. The meat portion of the diet was reduced by degrees daily (between April 22 and May 1) to 60 grams and the weight fell meanwhile from 8.39 to 8.3 kilos. On May 3 the daily portion of meat was decreased to 50 grams, and on the 7th of May to 40 grams. The weight had then fallen to 8.25 kilos. The daily diet contained 40 grams of meat from May 7 to the end of the experiment. This diet contained 1.943 grams of nitrogen (about 0.23 gram per kilo). Between May 7 and May 26, the weight fell from an average of 8.23 kilos to 8.14 kilos, but the dog's general condition remained excellent. She was active, playful and in general health could not be observed to suffer in any way as a result of the low diet.

First Hemorrhage. June 1, 11 a.m. The dog was put under ether narcosis, the right femoral artery was exposed, a cannula was inserted and 327 grams of blood were slowly withdrawn. At no time during the withdrawal, which lasted 11 minutes, were there any signs that the dog was in a critical condition. Consciousness returned about the time the hemorrhage was completed. The dog lay quietly on the table and apparently did not suffer. The wound was closed and the animal returned to the cage. At 5 p.m. she ate her food greedily and at 6 p.m. was quite lively and noisy. The amount of blood withdrawn was equal to 4.2 per cent of the body weight.

June 2. This morning the dog appears normal. Aside from a slight stiffness of the leg operated upon there is nothing to suggest yesterday's manipulation. (See Table XII.)

Second hemorrhage. June 5, 10:30 a.m. The left femoral artery was exposed under ether narcosis, a cannula was inserted and 236 grams of blood were withdrawn in the course of 10 minutes. During the latter part of the experiment there were signs of respiratory failure. The pulse could not be felt and the heart action was weak and rapid. On this account the experiment was discontinued and the wound closed. She recovered from the operation within 20 minutes and soon played about the cage and barked vigorously. The amount of blood withdrawn was equal to 3 per cent of the body weight. At 5 p.m. the dog took her food as usual and did not act in any way sick.

June 6. The dog is lively and playful.

June 7. Dog appears perfectly normal. (See Table XII.)

Third hemorrhage. June 9, 10 a.m. The right femoral artery was exposed on the proximal side of the former wound, a cannula was inserted and 227 grams of blood were withdrawn. After 12 minutes the dog appeared to be in a critical condition and the wound was closed. The amount of blood withdrawn was equal to 2.9 percent of the body weight. *5 p.m.* The dog took her food greedily. She appeared happy most of the day, is much quieter than usual, but does not act sick.

June 10. Dog has slept all day except when spoken to. She ate her food with apparent relish, and aside from acting rather logy shows no unusual symptoms.

June 11. Dog's appearance *almost normal* today.

June 12. Dog was lively and noisy.[1] (See Table XII.)

SIXTH HEMORRHAGE EXPERIMENT. *Twin dog on a* high *plane of protein nutrition. (Compare with the fifth hemorrhage experiment).*

This animal, the male Scotch terrier, was put on the following diet at the start: 144 grams of meat, 36 grams of cracker meal, 27 grams of lard, with 10 grams of bone ash and 350 cc. of water (April 9). With this diet the dog maintained practically a uniform weight of 8.8 kilos. On April 13 the the daily amount of meat was increased 10 grams and again the same amount on April 15 and April 17, making on the latter date 164 grams of meat in the diet. Between April 17 and April 22 the weight slowly rose from 8.8 to 8.9 kilos. On April 22 the meat in the diet was increased to 177 grams and the weight gradually increased to 9.1 kilos on May 17. The amount of meat was then raised to 194 grams and this ration was main-

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[1] The animals used in hemorrhage experiments five and six were in normal health until the autumn of 1909. After the completion of the experiments they were sent to a suburb as family pets. They had to be killed on account of exposure to rabies in 1909.

TABLE XII. (Compare directly with Table XIII).

FIFTH HEMORRHAGE EXPERIMENT. Low *plane of protein metabolism.*
FIRST PERIOD. BEFORE *hemorrhage.*

DATE 1908 MAY	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.	WEIGHT OF DRY FECES GRAMS.
27	8.18	265	16	3.58	0.25	3.83	0.21	0.06	0.27	16
28	8.12	215	13	2.90	0.29	3.19	0.17	0.07	0.24	19
29	8.15	260	16	3.51		3.51	0.21		0.21	
30	8.11	265	14	3.58	0.46	4.04	0.21	0.11	0.32	30
31	8.15	245	16	3.31	0.29	3.60	0.19	0.07	0.26	19
June.										
1	8.09	290	15	3.91	0.46	4.37	0.23	0.10	0.33	30
AVERAGE	*8.13*	*253*	*15*	*3.46*	*0.29*	*3.75*	*0.20*	*0.07*	*0.27*	*19*

SECOND PERIOD. AFTER *the* FIRST *hemorrhage (4.2 per cent of body weight)*

2	7.83	150	30	3.08		3.08	0.15		0.15	
3	7.84	200	20	4.11	0.39	4.50	0.21	0.10	0.31	25
4	7.90	205	14	3.00	0.28	3.28	0 17	0.07	0.24	18
5	7.99	215	16	3.14	0.34	3.48	0.18	0.09	0.27	22
AVERAGE	*7.89*	*192*	*20*	*3.33*	*0.25*	*3.58*	*0.18*	*0.06*	*0.24*	*16*

THIRD PERIOD. AFTER *the* SECOND *hemorrhage (3.0 per cent of body weight).*

6	7.75	125	29	1.79		1.79	0.11		0.11	
7	7.70	290	10	4.16		4.16	0.25		0.25	
8	7.75	260	11	4.12	0.65	4.77	0.32	0.13	0.45	42
9	7.80	110	35	1.74	0.31	2.05	0.14	0.10	0.24	20
AVERAGE	*7.75*	*196*	*21*	*2.95*	*0.24*	*3.19*	*0.20*	*0.06*	*0.26*	*15*

FOURTH PERIOD. AFTER *the* THIRD *hemorrhage (2.9 per cent of body weight).*

10	7.72	90	43	2.13		2.13	0.12		0.12	
11	7.56	210	19	4.96	0.35	5.31	0.28	0.12	0.40	23
12	7.58	170	19	2.44	0.34	2.78	0.13	0.11	0.24	22
13	7.69	160	10	2.29		2.29	0.13		0.13	
AVERAGE	*7.64*	*157*	*23*	*2.95*	*0.17*	*3.12*	*0.16*	*0.06*	*0.22*	*11*

TABLE XIII. (Compare directly with Table XII).

SIXTH HEMORRHAGE EXPERIMENT. High *plane of protein metabolism*
FIRST PERIOD. BEFORE *hemorrhage.*

DATE 1908 MAY	BODY WEIGHT KILOS.	VOLUME OF URINE CC.	SP. GR. OF URINE 1.0XX.	N OF URINE GRAMS.	N OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.	WEIGHT OF DRY FECES GRAMS.
27	9.18	320	25	8.15	0.60	8.75	0.48	0.16	0.64	31
28	9.13	270	25	6.88	0.70	7.58	0.40	0.19	0.59	36
29	9.21	220	33	5.60		5.60	0.33		0.33	
30	9.25	330	21	8.40	0.47	8.87	0.49	0.11	0.60	24
31	9.20	320	22	8.15	0.58	8.73	0.48	0.14	0.62	30
June.										
1	9.18	300	25	7.64	0.54	8.18	0.45	0.13	0.58	29
AVERAGE	*9.19*	*293*	*25*	*7.47*	*0.48*	*7.95*	*0.44*	*0.12*	*0.56*	*25*

SECOND PERIOD. AFTER *the* FIRST *hemorrhage (3.6 per cent of body weight).*

2	8.93	175	44	6.18	0.31	6.49	0.34	0.13	0.47	22
3	8.95	255	29	9.01	0.62	9.63	0.50	0.25	0.75	44
4	8.97	265	26	7.08	0.27	7.35	0.41	0.11	0.52	19
5	9.11	165	22	4.41	0.52	4.93	0.26	0.21	0.47	37
AVERAGE	*8.99*	*215*	*30*	*6.67*	*0.43*	*7.10*	*0.38*	*0.17*	*0.55*	*30*

THIRD PERIOD. AFTER *the* SECOND *hemorrhage (3.4 per cent of body weight)*

6	8.91	210	42	6.41	0.17	6.58	0.38	0.06	0.44	10
7	8.90	345	21	10.53		10.53	0.62		0.62	
8	8.93	245	25	7.60	0.67	8.27	0.43	0.23	0.66	39
9	8.93	235	34	7.29	0.46	7.75	0.41	0.16	0.57	27
AVERAGE	*8.92*	*259*	*30*	*7.96*	*0.32*	*8.28*	*0.46*	*0.11*	*0.57*	*16*

FOURTH PERIOD. AFTER *the* THIRD *hemorrhage (3.7 per cent of body weight).*

10	8.78	95	48	3.98		3.98	0.22		0.22	
11	8.83	210	40	8.81	0.53	9.34	0.48	0.18	0.66	31
12	8.88	275	26	7.31	0.92	8.23	0.42	0.32	0.74	54
13	8.98	185	22	4.92	0.46	5.38	0.29	0.16	0.45	27
AVERAGE	*8.87*	*191*	*34*	*6.25*	*0.48*	*6.73*	*0.35*	*0.16*	*0.51*	*28*

tained throughout the remainder of the experiment. This diet contained 7.532 grams of nitrogen (0.81 gram per kilo. considering the average weight for the preliminary period to be 9.19 kilos). The dog appeared to be in perfect health. While not so lively as the twin dog in the fifth hemorrhage experiment, he was always playful and happy.

First hemorrhage. June 1, 1908, 10:30 a.m. The right femoral artery was exposed under ether narcosis, a cannula was inserted and 331 grams of blood were withdrawn. The hemorrhage was stopped at this point in the withdrawal because the respirations indicated a critical condition, and heart action was bad. The dog took the ether badly at the beginning of the experiment and, after the termination of the hemorrhage, artificial respiration was necessary for some minutes. Period of hemorrhage, 10 minutes. The amount of blood withdrawn was equal to *3.6 per cent* of body weight. The dog became conscious one-half hour after being returned to the cage. and at 12 o'clock he was walking about but did not act in a normal condition. He ate his regular meal at 5 p.m. At 6 p.m. he appeared active, barked when he was spoken to and did not in any way act sick.

June 2. The dog is apparently normal. (See Table XIII).

Second hemorrhage June 5, 10 a.m. The left femoral artery was exposed under ether narcosis, a cannula was inserted and 312 grams of blood were slowly withdrawn in the course of 12 minutes. Respiration and pulse began to fail and the hemorrhage was discontinued at this point. The amount of blood withdrawn was equal to *3.4 per cent* of the body weight. The dog recovered from the influence of the ether in 15 minutes, and then walked about the cage. He did not act particularly weak and showed no serious effects of the hemorrhage. *5 p.m.* He ate all his food greedily. *6 p.m.* He was active, and jumped about and barked when spoken to.

June 6. No discernible signs of yesterday's hemorrhage.

June 7. The dog acts perfectly normal. (See Table XIII).

Third hemorrhage. June 9, 10 a.m. The dog was put under ether narcosis, the right femoral artery was exposed and cannulized, and 332 grams of blood were slowly withdrawn. There was no apparent respiratory distress and the hemorrhage was discontinued because the blood would no longer run from the cannula although there apparently was no clot or other obstruction. The period of withdrawal was 11 minutes. The amount of blood was equal to 3.7 per cent of the body weight. *11 a.m.* The dog is lying quietly in his cage, but acts sleepy and logy. No cardiac or respiratory distress apparent. *3 p.m.* The dog ate all his food. Walks about his cage, acts weak but not sick.

June 10. The dog is very quiet. Sleeps most of the time which is unusual for this animal. No other symptoms of an abnormal condition.

June 11. The dog is more lively today than yesterday, but is not yet as active as normal.

June 12. The dog is very noisy and playful this morning. There is apparently nothing in his general condition that is abnormal.[1] (See Table XIII).

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[1] See the footnote on page 413.

TABLE XIV.

A summary of Average metabolic data, per period, for the hemorrhage experiments (1-6).

FIRST EXPERIMENT. Low *plane of protein metabolism.*

		BODY WEIGHT KILOS.	VOLUME OF URINE CC.	N OF URINE GRAMS.	OF FECES GRAM.	TOTAL N GRAMS.	S OF URINE GRAM.	S OF FECES GRAM.	TOTAL S GRAM.
Before hemorrhage		7.64	270	2.51	0.24	2.76	0.16	0.06	0.23
After hemorrhage	(3.38%)	7.29	223	3.65	0.31	3.96	0.24	0.08	0.32

SECOND EXPERIMENT. High *plane of protein metabolism.*

Before hemorrhage		6.00	290	6.05	0.37	6.42	0.37	0.09	0.46
After 1st hemorrhage	(4.31%)	5.76	275	6.68	0.42	7.10	0.41	0.10	0.51
After 2nd hemorrhage	(4.3 %)	5.76	247	6.10	0.30	6.41	0.39	0.06	0.46
After 3rd hemorrhage	(3.1 %)	5.70	289	6.62	0.30	6.93	0.41	0.06	0.48

THIRD EXPERIMENT. High *plane of protein metabolism.*

Before hemorrhage		12.83	300	12.99	0.62	13.61	0.85	0.18	1.03
After 1st hemorrhage	(3.8 %)	12.14	300	13.43	0.56	14.00	0.80	0.23	0.98
After 2nd hemorrhage	(2.3 %)	12.07	217	10.85	0.61	11.47	0.73	0.18	0.92
After 3rd hemorrhage	(0.7 %)	11.78	223	10.18	0.49	10.67	0.60	0.14	0.74

FOURTH EXPERIMENT. Low *plane of protein metabolism.*

Before hemorrhage		6.11	249	2.42	0.20	2.62	0.13	0.05	0.18
After 1st hemorrhage	(4.02%)	5.89	218	2.38	0.17	2.55	0.17	0.06	0.23
After 2nd hemorrhage	(4.0 %)	5.64	224	2.50	0.18	2.68	0.14	0.06	0.20
After 3rd hemorrhage	(3.9 %)	5.48	207	2.55	0.27	2.82	0.16	0.09	0.26

FIFTH EXPERIMENT. (*Twin female*). Low *plane of protein metabolism*

Before hemorrhage		8.13	253	3.46	0.29	3.75	0.20	0.07	0.27
After 1st hemorrhage	(4.2 %)	7.89	192	3.33	0 25	3.58	0.18	0.06	0.24
After 2nd hemorrhage	(3.0 %)	7.75	196	2.95	0.24	3.19	0.20	0.06	0.26
After 3rd hemorrhage	(2.9 %)	7.64	157	2.95	0.17	3.12	0.16	0.06	0.22

SIXTH EXPERIMENT. (*Twin male*). High *plane of protein metabolism.*

Before hemorrhage		9.19	293	7.47	0.48	7.95	0.44	0.12	0.56
After 1st hemorrhage	(3.6 %)	8.99	215	6.67	0.43	7.10	0.38	0.17	0.55
After 2d hemorrhage	(3.4 %)	8.92	259	7.96	0.32	8.28	0.46	0.11	0.57
After 3d hemorrhage	(3.7 %)	8.87	191	6 25	0.48	6.73	0.35	0.16	0.51

GENERAL REVIEW.

It is evident that results so equivocal as the foregoing must be interpreted most conservatively. In the first series of experiments with ricin six dogs were used. Three of these dogs had been fed on relatively large amounts of meat and all died as the result of the inoculations. Of two dogs on low planes of protein nutrition, one died and one survived. It does not seem probable that the diet rich in protein was in any degree responsible for the mortality of the dogs on the high protein planes, because the doses of ricin were computed on the weight of the animal and these dogs, having taken on weight as a result of liberal feeding, received relatively larger doses of the toxin than did the dogs that were poorly nourished. That ricin is unsuitable for a research of this sort is evidenced by the fact that any individual peculiarities in combating the toxemia are entirely obscured by the severity of the symptoms induced.

In the work on the effects of hemorrhage there are several considerations of interest. The mortality of the animals on low planes of protein nutrition was the same as that of the well fed; one dog in each series succumbed (first and third hemorrhage experiments). The dog that died while on the high plane of protein nutrition (III) was the collie whose peculiar condition and behavior has already been noted, and in this instance death was not a surprise, but in the case of the dog on the low plane of protein nutrition (I) a fatal termination was unexpected. Comparison with other observations shows that animals on higher planes of protein nutrition (second experiment) and also on lower planes (fourth experiment) withstood more severe losses of blood, in the case of the latter animal each loss of blood amounted to 4 per cent of the animal's weight. The data in table XIV suggest that the amounts of food were not so important in determining the behavior of the animals after hemorrhage as some other factors, perhaps the breed of the dogs. While this point does not appear in the protocols it was evident to one observing the various dogs daily. It was a most striking fact that certain types of these animals bore the operation well, recovered quickly and showed surprisingly few effects of the blood loss, and this without regard to the plane of protein nutrition induced in these experiments. In some

degree, at least, this impression is supported by the hemorrhage experiments with the twins, (fifth and sixth experiments). In these animals no effects were observed, either before or after the bleedings, that could be ascribed to the differences in their respective states of nutrition. The female (fifth hemorrhage experiment) was at first more active than the male, and at no time during the experiment did the low diet produce a change in this respect. The recovery of the male (sixth experiment) after the hemorrhages was less prompt at first than in the case of the female, but this was in entire accord with the animals' behavior in other respects.

Finally, the impression made on the observer by these studies was that within relatively wide limits the total amount of food, as well as its total content of protein, were less important factors in determining the animal's resistance in these particular experiments than the peculiar cellular processes which, for lack of a better and more definite term, may be called individual idiosyncrasy.

This study was begun during the summer of 1905, at Dr. Gies' suggestion. It has been carried forward under his direction and with the aid of a grant from the Rockefeller Institute for Medical Research.

A NEW METHOD FOR THE DETERMINATION OF FAT AND FATTY ACIDS IN FECES.

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An adequate knowledge of fat metabolism is particularly important as a prerequisite for a rational treatment of the numerous cases of digestive disorders occurring among infants and young children. Without reasonably reliable methods for determining what can properly be called fat in the stools such knowledge is scarcely obtainable. In the course of several years' study in this field involving many fat metabolism experiments, one of us (Wentworth) has gradually come to see that the methods in use for the determination of the different fat constituents in feces are not satisfactory.[1] We believe that the method described in this paper, simple as it is, meets the needs of the situation.

In working out this method we have had two important considerations in mind. First, that everything which can be taken out by means of organic solvents from dried stools is not fat; secondly, that it is practically impossible to determine separately the free fatty acids and the soaps.

That a solvent like chloroform will take out substances which are not fat or fatty acids is well known, but it appears to be less well known to what extent the substances so extracted interfere with the determination. After extracting dried stools with anhydrous ether for twenty hours and then for another twenty hours with ether acidified with hydrochloric acid gas, we have found that chloroform will still take out relatively large quantities of

[1] This statement refers only to the extraction methods ordinarily in use. With the recent method of Kumagawa and Suto (*Biochemische Zeitschrift*, ix, p. 337, 1908) we have had no experience.

a mixture possessing an intense fecal odor. Since fat and fatty acids are readily soluble in all the ordinary organic solvents, and since there are other constituents in feces which are soluble in some such solvents, it would appear more or less self-evident that the solvent to be used should be selected with reference to how little it will take out rather than with reference to how much. We conclude from our experience that the use of chloroform is not permissible, for even with the subsequent treatment of the chloroform extract with petroleum ether it is not possible to separate the fat and fatty acids from the impurities. For special reasons which will be given later we use anhydrous ether for our extractions and then purify the extract by treatment with low boiling petroleum ether (ligroin).

With reference to the separate determination of the soaps and fatty acids there is this to be said. The ordinary fatty acids are extremely weak acids and the extent to which they will combine with bases to give soaps in such a complex mixture as the stools is not very important. Numerous different factors of no special bearing on fat metabolism will materially effect the equilibrium between the soaps and fatty acids. The water, the hydrogen sulphide, the carbon dioxide, the calcium and magnesium, to say nothing of the heat and ammonia formation during the drying of the stools, all affect the soaps. The proportion of free fatty acid extracted by means of neutral organic solvents from the dried stools bears, therefore, no definite relationship to the proportion as it existed at the time of defecation or at any time previous to the defecation.

In view of these considerations we set ourselves the task of determining (*a*) the total fat including neutral fat and total fatty acid; (*b*) the total fatty acid including the fatty acids of the soap. The neutral fat is obtained by the difference between the two results.

Having found that anhydrous ether acidified with hydrochloric acid gas readily takes out the fatty acids from soaps, we now accomplish the determination by means of a single extraction. And having discovered a method by means of which even the higher fatty acids can be titrated as sharply as the strongest mineral acids, the determination involves but one weighing and one titration.

Our titration method represents, we believe, a new departure in alkalimetry and is not only interesting from a theoretical standpoint but practically should become applicable to a large number of organic acids which hitherto it has not been possible to determine by titration.

The method is based on the use of sodium alcoholate as the alkali and certain organic solvents such as chloroform, carbon tetrachloride, benzol or toluol as solvent for the acid which is to be titrated. Sodium alcoholate and certain organic solvents have of course been used before in titrations but so far as we know, none of the above mentioned solvents have been used. The organic solvents which have been employed, alcohol, ether or acetone, are almost useless. It is, in fact, largely because of the astounding differences in the quality of the end-point of the titrations obtained by means of these two sets of solvents that we have concluded that the availability of the first named set has until now remained undiscovered.

Organic solvents are held to be much less desirable than water. The ordinary oxygen-containing organic solvents act like weak acids with an indicator like phenolphtalein, consequently a considerable excess of alkali is used up and the end-point obtained is extremely uncertain. These phenomena are explained on the basis of the associating effects of the organic solvents as against the dissociating effects of water. With the above-mentioned oxygen-free solvents, no such "associating" results are obtained. Further the addition of alcohol, ether or acetone to these solvents is as disastrous to the end-point of the titration as it is in the case of water.

These phenomena are being further investigated in this laboratory with reference to the different organic solvents and indicators and their applicability to the titration of a number of organic acids, hence it is unnecessary to discuss the matter any further in this paper. Here we wish only to emphasize the empirical finding that on titrating a higher fatty acid like stearic acid in hot solutions of benzol or carbon tetrachloride, theoretical figures are obtained and that the end-point, a deep purple, is obtained with the addition of the last 0.05 cc. of the decinormal sodium alcoholate solution.

The sodium alcoholate solution is prepared by dissolving about

2.3 grams of metallic sodium in about 1 liter of absolute alcohol It is easily and quickly prepared and can be standardized in the usual manner against decinormal hydrochloric acid. The coating of oxide which always covers sodium sticks should of course be scraped off or cut away before the sodium is weighed.

The ethereal hydrochloric acid solution used for the extraction should be decinormal or a little stronger. It is conveniently prepared by dropping concentrated sulphuric acid on about 10 grams of powdered sodium chloride and leading the gas into about 1 liter of ether. Cold ether holds hydrochloric acid fairly well and that is why we use this solvent by preference for the extractions. The ether must be of the absolute sodium dried variety as both water and alcohol must be carefully excluded. The hydrochloric acid content of the ether is determined by titration (in the presence of water) and is then diluted to the desired concentration by the addition of more anhydrous ether.

The determination is carried out as follows: The thoroughly dried stool is pulverized and sifted through a 40 mesh sieve. All should go through the sieve. The whole is then sifted through once more in order to insure thorough mixing of the sample. The thorough powdering of the stools is an important detail, for without it it is well nigh impossible to obtain complete extraction.

One gram of the powder is then weighed out, wrapped up in a piece of fat-free filter paper and the whole transferred to a fat-free filter paper "thimble." This is inserted in the extraction apparatus which is then attached to a 250 cc. Erlenmeyer flask containing about 150 cc. of the ethereal hydrochloric acid solution. The boiling of the ether should then be kept up for about 20 hours. After disconnecting the flask the ether is distilled off. With it goes the hydrochloric acid, provided that no alcohol or water is present. (Any traces of HCl which may remain are removed during the ligroin treatment). When practically all the ether has been thus removed about 50 cc. of low boiling petroleum ether is added and the flask is set aside over night. The petroleum ether should have a boiling point of 30 to 60° C., i.e., when distilled all should go over below 60°.

The following day the petroleum ether solution is filtered through a small plug of absorbent cotton inserted in the stem of

a suitable funnel or "adapter." The filtrate and washings are collected in a weighed, tall, 100 cc. beaker. The solvent is boiled off, the residue is dried at about 95° C. for five hours, cooled and weighed. This gives the total weight of the neutral fats and the fatty acids.

The fatty residue is then dissolved in 50 cc. of benzol, one or two drops of a 0.5 per cent alcoholic solution of phenolphtalein is added, and the mixture is heated until the boiling point is nearly reached. It is then immediately titrated with the standardized sodium alcoholate solution. The titration should be continued until the maximum color of the indicator is obtained. The subsequent more or less rapid fading away of the color does not indicate that the true endpoint was not reached. This fading seems to be due mainly to the fact that on cooling the soap which is formed is transformed into basic soap, thereby setting free a little of the acid.

Each cubic centimeter of decinormal alkali solution used corresponds to 28.4 mgm. of stearic acid and all our results thus far obtained indicate that the fatty acid in stools consists mainly of stearic acid.

The following results obtained on the stools of normal adults were obtained by the method described above:

Per Cent	1	2	3	4	5	6
Total fat	13.5	16.0	17.3	23.5	20.0	29.2
Neutral fat	6.8	6.6	6.6	8.5	7.7	5.4
Fatty acid	6.7	9.4	10.7	15.0	12.3	23.8

While the total fat varies between 13 and 30 per cent in round numbers, the neutral fat varies only between 5 and 9 per cent. In other words the larger the amount of total fat the feces contains, the more of that fat is present as fatty acids (including the soaps).

In the case of an "atrophied" infant the results obtained are even more striking, for here the total "fat" seems to consist almost entirely of fatty acids and soaps.

The following figures were obtained: On modified cow's milk, total fat 54.5 per cent, fatty acid 54.6 per cent. On mother's milk, total fat 28.8 per cent, fatty acids 25.8 per cent.

It may be thought that these high fatty acid values are due to the splitting effect of the acid ether, but such is not the case. By special experiments we have satisfied ourselves that neutral fats are not saponified by being boiled with decinormal solutions of hydrochloric acid in anhydrous ether. Neither can the figures be explained on the basis of lower fatty acids for the extracts melted at about 53° C., a figure easily accounted for on the basis of a small admixture of oleic acid.

CARBOHYDRATE ESTERS OF THE HIGHER FATTY ACIDS.

(Preliminary Paper.)

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In a recent paper[1] Neuberg and Pollak reserve the field for the study of the carbohydrate esters of phosphoric acid and in view of the nearness of their work to that upon which I am now engaged this brief preliminary paper, part of the thesis to be presented as a doctor's dissertation, is presented to ensure undisturbed possession of this field for a time. My investigations, undertaken at the suggestion of Professor Folin, deal with the preparation of the carbohydrate esters of the higher fatty acids and the behavior of such compounds in the animal organism. In looking over the literature it was found that no serious attempt had been made to prepare and study any such compounds. Berthelot[2] had described briefly a number of substances obtained by heating together various carbohydrates and fatty acids to high temperatures for several hours, but his yields were small and as he made no effort to determine the purity of his compounds, his contribution gives but slight help in the solution of this problem.

The method of synthesis which seemed best adapted to the work in hand was the familiar sulphuric acid esterification method which has been successfully used by Grün[3] in the synthesis of the glycerides. For the first part of the work mannite was used because of its stability under the conditions of the experiment

[1] *Biochem. Zeitsch.*, xxiii, p. 515.

[2] Berthelot: *Ann. de chim. et de phys.*, (3), lx, p. 95.

[3] Grün: *Ber. d. deutsch. chem. Gesellsch.*, xxxviii, p. 2284; xl, pp. 1778 and 1795.

and because it is readily utilized by the organism. The fatty acid used was stearic acid. The procedure was as follows: 10 gm. of mannite was gently warmed with 75 to 100 cc. of concentrated sulphuric acid until solution had taken place, 30 gm. of stearic acid added and the whole stirred until the acid was dissolved. The clear, brown mixture was then heated for three hours to 65° to 75°, at the end of which time the liquid had darkened in color, but was still clear. It was cooled and extracted with ether as directed by Grün.[1] In this process considerable heat was developed and the ether boiled vigorously for a short time, while the pasty mass increased in volume and became liquid. At first not much attention was paid to this phenomenon and as considerable ether was used the heating and liquefaction were thought to be due to the moisture in the ether. Later, when one of the carefully purified compounds was found to be ethyl stearate, attention was forcibly recalled to this reaction. The explanation is that ether dissolves in concentrated sulphuric acid to form ethyl sulphuric acid.[2] This with the uncombined stearic acid in the mixture forms ethyl stearate which dissolves in the ether along with the mannite compounds. Since its properties are quite like those of some of the mannite derivatives its presence makes the purification of these substances exceedingly difficult. Of course the longer the mixture stands before the sulphuric acid is washed out, the greater will be the amount of ethyl stearate formed; and as Grün makes no mention of the presence of such a compound it is to be presumed that he has overlooked it in his work. He makes no statement as to the length of time allowed for his extractions. In my experiments the ether was allowed to stand in contact with the mixture over night[3] to ensure complete extraction. The combined ether extracts contained, at this

[1] Grün: *Loc. cit.*

[2] Beilstein: *Org. Chem.*, i, p. 294.

[3] To show that ethyl stearate is formed under these conditions, some stearic acid was dissolved in concentrated sulphuric acid, heated three hours as in the above experiment, cooled, shaken out with several volumes of ether and allowed to stand in contact with the ether over night. The ether was poured off, washed with water, the dissolved fatty acid removed as soap and the ether evaporated. The yield of ethyl stearate under these conditions was about 15 per cent of the fatty acid used.

point, a voluminous white suspension, which was filtered off and saved for later examination. The ethereal filtrate was washed several times with cold water and the fatty acid removed by titration with $\frac{N}{1}$ alcoholic soda solution and filtration of the precipitated soap. The ether was then distilled off and the product purified by fractional precipitation from methyl alcohol until the precipitate and the portion dissolved in the mother liquor gave the same melting point. This required in many cases as many as twenty-five separations.

The substance so obtained is pure white, semi-translucent, brittle and amorphous, breaking with conchoidal fracture. It is obtained with difficulty in the crystalline condition from ether at 5°C. as balls of closely packed microscopic needles. It is insoluble in water, slightly soluble in cold methyl and ethyl alcohols, readily soluble in them hot, soluble in cold ether, benzol and chloroform,—the solubility increasing in the order named. It melts at 51°C. It is heavier than water. It is optically active, its rotation determined in benzol being as follows:

(1) 0.677 gm. in 10 cc. benzol in 1 dm. tube = +4.39°
(2) 0.772 gm. in 10 cc. benzol in 1 dm. tube = +5.02°

$$(1)\ [\alpha]^{20}_{D} = 64.84^\circ \quad (2)\ [\alpha]^{20}_{D} = 63.03^\circ$$

$$\text{Average } [\alpha]^{20}_{D} = 63.9^\circ$$

It is readily saponified by boiling with alcoholic soda, yielding stearic acid and a brown syrup from which in time colorless flat plates separate. These were identified as mannite by dissolving out the syrup with absolute alcohol, recrystallizing from water, and determination of the melting point.

Determination of the fatty acid content by saponification and titration of the purified fatty acid gave the following figures:

(1) 0.305 gm. of the ester gave 0.2562 gm. of stearic acid
(2) 0.3033 gm. of the ester gave 0.2601 gm. of stearic acid
Per cent stearic acid (1) 84; (2) 85.7 Average, 84.8.

Molecular weight determinations by the cryoscopic method from benzol resulted as follows:

(1) 0.3962 gm. in 8.68 gm. benzol	Lowering = 0.32°
(2) 0.592 gm. in 8.68 gm. benzol	" 0.493°
Molecular weight, (1) 712; (2) 700	Average, 706.

The analyses correspond most closely to mannid distearate, of the formula $C_6H_8O_2$ $(C_{18}H_{35}O_2)_2$, having a stearic acid content of 83.8 per cent and a molecular weight of 678. That mannite is dehydrated to mannid in the process of esterification where the conditions are favorable for dehydration, has been definitely shown by Carré[1] in his work on the mannite esters of phosphoric acid.

A thorough study of the reaction outlined above is in progress and the work will then be extended to the true carbohydrate compounds of the fatty acids.

[1] Carré: *Ann. de chim. et de phys.*, (8), v, pp. 345–432.

THE PRODUCTION OF VOLATILE FATTY ACIDS AND ESTERS IN CHEDDAR CHEESE AND THEIR RELATION TO THE DEVELOPMENT OF FLAVOR.

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Investigators of the chemical changes taking place in the process of cheese ripening have concerned themselves almost wholly with the nitrogenous side. The changes which the proteins undergo in the curing process, and the nitrogenous cleavage products formed have been studied by a number of investigators. Contributions to this phase of the subject have been made by Winterstein[1] on Emmenthaler cheese, by Babcock and Russell[2] and Van Slyke and Hart[3] for Cheddar cheese, and Dox[4] for the soft Camembert cheese. To these bodies, in part at least, have been attributed the cheese flavor.

Our own investigations on the failure of inherent enzymatic proteolysis alone in a curing Cheddar cheese to adequately account for the development of typical cheese flavor, led us to this study of the non-nitrogenous products formed during the normal curing process. This paper deals only with American Cheddar cheese.

According to present views, the factors involved in the curing of Cheddar cheese are the pepsin contained in the rennet; the

[1] Beiträge zur Kenntniss der Bestandtheile des Emmenthalerkäses, *Zeitschr. f. physiol. Chem.*, xxxvi, pp. 41 and 47.

[2] Unorganized Ferments of Milk, a New Factor in the Ripening of Cheese, Wis. Agr. Exp. Sta., 14 Annual Rept. for 1897.

[3] A Contribution to the Chemistry of American Cheddar Cheese, *Amer. Chem. Journ.*, xxix, 1903.

[4] Proteolytic Changes in the Ripening of Camembert Cheese, Bureau of Animal Industry, U. S. Dept. of Agr., Bul. 109.

activating lactic acid formed from lactose fermentation; galactase, the inherent proteolytic enzyme of milk; and some biological factor other than that simply concerned in the lactose fermentation. The last, at the present state of our knowledge, is practically undefined.

There are constantly found in milk three classes of acid-forming organisms: (1) the group of bacteria represented by *B. lactis acidi*, (2) the aërogenes group, represented by *B. coli communis* and *B. lactis aërogenes*, (3) a less well defined group, the members of which are cocci, varying in color from white through the various shades of yellow to orange. These organisms may or may not liquefy gelatin, and vary greatly in their ability to produce acid. The groups of acid-forming organisms differ not only in morphology, in cultural characteristics, but especially in the products formed in their fermentation of milk.

The spontaneous souring of milk is due to the combined action of these three groups of bacteria. The relative number of bacteria belonging to these groups will vary widely from one sample of milk to another, being influenced by a large number of factors, such as cleanliness in milking, temperatures at which the milk is kept, etc. It is undoubtedly true that the majority of the bacteria in good milk ripened to a point desired for the making of Cheddar cheese, belong to the first group. If the milk is of poorer quality, the number of gas-forming organisms, the second group, will be greater. They are, however, always present in milk. The third group is likewise constantly present, as can be shown by appropriate means, but nothing is known of their relative numbers in milk.

These various organisms are enclosed in the curd and undoubtedly form the same products as when growing in milk. A number of investigators have studied in greater or less detail the by-products of the acid fermentation of milk by pure cultures of representatives of the first two groups mentioned. The organisms of the first group produce from 90 to 98 per cent of the theoretical yield of lactic acid from the sugar fermented. The remaining portion of the sugar appears in the form of other by-products, among which have been found alcohols, aldehydes and esters, whose presence indicates the formation of volatile fatty acids.

With the second group, the aërogenes-colon group, the lactic

acid may not exceed 30 per cent of the total acid formed, the remaining being largely acetic, succinic, and formic acids, with some alcohols, and in addition such gases as carbon dioxide, hydrogen and methane. The difference in taste between milk soured by a mixture of organisms, as in the spontaneous souring of milk, and milk soured by a pure culture of *B. lactis acidi*, emphasizes the variation in the products other than lactic acid.

Nothing is known concerning the products of the third group of organisms. It is evident that during the initial fermentation of the sugar in the cheese, a considerable number of products will be formed in varying amounts, depending upon the proportion existing between the groups of acid-forming organisms present. It was thought that these various products might, through decomposition, or by combination with substances formed later in the curing of the cheese, play an essential rôle in the development of flavor.

It was also believed not improbable that the lactic acid formed in the early process of Cheddar cheese making remained only as an intermediate product and was by no means the final product, but suffered further decomposition and rearrangement. The possibility of its being the mother substance of certain volatile fatty acids, which, by further combination with alcohols and production of esters, contribute to flavor production, formed the basis of our working hypothesis.

Jensen[1] has studied the occurrence of volatile fatty acids in Emmenthaler and also certain other kinds of European cheese, but his work did not involve a measure of the progressive accumulation or disappearance of such bodies during a long extended curing process. Our own work involved, at definite periods of the curing process, as complete an analysis of the cheese as available methods would allow. Quantitative estimations of lactic and volatile fatty acids, such as acetic, propionic, butyric, caproic and formic acids, were made. Qualitative separation of certain hitherto unknown bodies, such as esters, with identification of the acids and alcohols forming them, was also made at definite stages of the ripening period. In addition qualitative isolation and identification of succinic acid were made, which, we believe, has never before been reported in curing Cheddar cheese.

[1] *Landw. Jahrbuch d. Schweiz*, XVII, p. 315. 1904.

EXPERIMENTAL PLAN.

A normal and a skim-milk American Cheddar cheese were made on August 1, 1908, and used for this investigation at definite intervals. Both cheeses were made from the same milk. The normal cheese was scored at the age of three months, and found to be in every way a marketable product. They were both cured at 55° F.

Description of Methods Used in the Chemical Analysis.

1. *Moisture determination.* About 10 grams of ground material were dried over sulphuric acid to constant weight. This required about ten days.

2. *Determination of lactic and separation of succinic acids.* Fifty to 100 grams of cheese were ground with pure quartz sand, strongly acidified with sulphuric acid and extracted with 75 to 100 cc. portions of ether about 24 times. The ether extract was evaporated to remove the ether, and in the case of the whole milk cheese, this ether extract residue was treated several times with hot water in a separatory funnel for the purpose of separating the fat from the extracted acids. The solution of acids was next boiled with an excess of barium hydroxide, then neutralized with dilute sulphuric acid and the precipitate removed. The filtrate and washings were evaporated to a small volume. The difficultly soluble barium succinate rapidly crystallized out. The crystals were collected and washed with 60 per cent alcohol, in which strength barium lactate is easily soluble. The mother liquor and washings from the barium succinate were evaporated and then diluted with water. Zinc sulphate was next added, care being taken to avoid an excess. Barium sulphate was removed and the filtrate evaporated until the first crystals of zinc lactate appeared. The solution was then placed in a dessicator and reduced to a small volume for further crystallization. The crystalline zinc lactate was collected and washed with a very small volume of cold water. The mother liquor and washings were treated in the same way for further crystallization. The united crystallizations were dried over sulphuric acid and quantitatively weighed. While the method is not absolutely quantitative, due to the slight solubility of the zinc salts of both inactive and active lactic acids, nevertheless by careful manipulation satisfactory results can be obtained.

Where determinations of the form of optical activity of the acids were made, definite portions were dried in an air oven at 108 to 110° C. for two hours. At a higher temperature, 115 to 120° C., there appeared to be some decomposition of zinc lactate.

3. *Determination of volatile fatty acids.* 250 to 300 grams of cheese were ground with sand, moistened with a dilute solution of sulphuric acid until the pasty mass gave a decided acid reaction to Congo red paper.

The mass was then distilled with steam. Usually a distillate of 1000 cc. was sufficient, but in certain analyses, as high as 2000 cc. were collected. The distillate was neutralized with barium hydroxide and again redistilled, in order to separate any neutral volatile substances, as alcohols and esters, that may be formed during the curing process. This fraction was concentrated through several redistillations: as for example, the first 1000 cc. of the steam distillate after neutralization with barium hydroxide, was distilled to 500 cc.; 500 cc. to 250 cc.; 250 to 150 cc.; and the 150 to 75 cc. This final and more concentrated fraction is here designated "flavor solution" because of the strong resemblance to the cheese aroma.

By the above procedure, two solutions are obtained, the neutral solution of the barium salts of the fatty acids and the so-called "flavor solution."

The former was evaporated to a volume of 150–200 cc. and after cooling, 20 to 30 cc. of $\frac{N}{10}$ sulphuric acid were added. The barium sulphate was filtered off and washed with cold water. The filtrate and washings were distilled to separate the free fatty acids. The first distillation is designated Fraction I. By repeating this process, a second fraction, F. II, and third fraction, F. III, were obtained. To these fractions the Duclaux[1] method for the estimation of fatty acids was applied.

O. Jensen[2] has already used this method with interesting results in his studies of foreign cheese.

The Duclaux method depends upon the fact that there is a constant rate of vaporization for each volatile fatty acid. For instance, when a definite amount of a solution of any volatile fatty acid is distilled, the amount of acid in each successive fraction of the distillate has its own constant proportion to the total acidity of the distillates. Duclaux distilled 110 cc. of the acid solution from a retort of 250 to 300 cc. capacity and titrated every 10 cc. of the successive distillations with standard lime water, until 100 cc. of the distillate were obtained. Each titre of lime water was calculated to per cent of the total titre of the 100 cc. of distillate. These per cents were of practically constant value for each of the volatile fatty acids.

In a mixture of several acids, each retains its own characteristic rate of vaporization. Because of this fact, the Duclaux method allows a quantitative estimation and identification of the volatile fatty acids contained in a solution.

Treatment of the "Flavor Solution."

The "flavor solution" was saponified with potassium hydroxide as follows: to 50 cc. there were added 10 cc. of 20 per cent potassium hydroxide and the mixture heated for two to three hours in a tightly stoppered pressure bottle at the temperature of boiling water. After cooling, the contents of the bottle were diluted and distilled in order that any alcohols, either in ester combination or free, might be collected. The residue from this distillation was acidified with dilute sulphuric acid and distilled with

[1] E. Duclaux: *Traité de microbiologie*, iii, p. 388, 1900.
[2] *Loc. cit.*

repeated additions of water. This distillate, containing volatile fatty acids, which had existed in ester combination, was neutralized with barium hydroxide and evaporated to a small volume. Barium was separated with dilute sulphuric acid and the solution of free fatty acids treated according to Duclaux' method for their identification.

The solution of alcohols from the saponification was oxidized as follows: 10 grams of potassium bichromate, and 20 grams of strong sulphuric acid were made up to 100 cc.; 25 cc. of this solution were added to about 50 cc. of the alcohol solution, contained in a tightly stoppered pressure bottle, and oxidation carried on at the temperature of the water bath for 2 to 3 hours. The excess of chromic acid was removed by zinc in the presence of sulphuric acid. The volatile fatty acids were next distilled as in all previous descriptions, using Duclaux' method for identification. All reagents used in the saponification and oxidation reactions were checked for freedom from volatile acid substances. In this respect, the chemicals used were pure.

Analysis of the Cheese at Different Ages.

A consideration of the work in greater detail will be given at this place, but will suffice for reference to all of the other periods.

Three-days old. For the determination of milk sugar, 25 grams of the cheese were ground with sand and extracted with hot water. The extracts were treated according to the method proposed by Scheibe[1] for the estimation of lactose. When the cheeses were three days old there was no reduction of the copper solution, showing that the sugar had all disappeared. In this instance, the disappearance was particularly rapid. In some previous work on the rapidity of sugar disappearance in fresh curds, lactose could be detected at the end of the third and even fifth day. It is very probable that the temperature and exposure to which the milk has been subjected prior to cheese making, would be an important factor in determining the rate of sugar disappearance in the manufactured curd.

Determination of the form of lactic acid in both whole and skim-milk cheese at the three day stage showed that it was of the inactive variety. From the whole milk cheese 0.390 gram of zinc lactate gave 0.069 gram of water of crystallization, equivalent to 17.85 per cent, while the theoretical amount for inactive zinc lactate is 18.18 per cent. From the skim-milk cheese 0.600 gram of zinc lactate gave 0.1045 gram of water of crystallization, equivalent to 17.41 per cent.

[1] *Zeitschr. f. analyt. Chem.*, xl, p. 13, 1901.

In the determination of volatile fatty acids, 250 grams of the whole milk cheese were ground, acidified and distilled to 1000 cc. The total acidity of the 1000 cc. was equivalent to 22.1 cc. of $\frac{N}{10}$ barium hydroxide. The neutral solution of the barium salts of the volatile fatty acids was evaporated to about 100 cc. acidified and fractionally distilled.

$$\text{Fraction I. } 130 \text{ cc.} = 6.40 \text{ cc. } \tfrac{N}{10}\, Ba(OH)_2$$
$$\text{" II. } 150 = 15.02 \text{ " " "}$$

As 10 cc. had been used in the determination of total acidity, the sum for the above fractions, corrected to 1000 cc., is equivalent to 21.60 cc. It is uncertain whether the difference of 0.5 cc. in acidity of the steam distillate and the sum of the fractions as shown above, was due to loss in manipulation or removal of barium carbonate by evaporation and filtration.

For the purpose of illustrating more in detail the method used in separating and identifying the volatile acids, data secured in the first analysis are displayed below. Tables of constants from which the calculations were made are available in Duclaux' *Trait de Microbiologie*, iii, 1900.

Fractions I and II treated according to Duclaux' method.
Fraction I = 130 cc. and required *6.4 cc.* $\frac{N}{10}$ *Ba(OH)*$_2$

DISTILLATE.	$Ba(OH)_2$.	FOUND.	CALCULATED FOR {A* = 79.15, P = 5.00, B = 15.83}
cc.		per cent.	per cent.
10	0.46 cc. × $\frac{100}{4.64}$ =	9.91	8.28
20	0.91 " " "	19.60	18.54
30	1.32 " " "	28.45	27.80
40	1.72 " " "	37.07	37.17
50	2.15 " " "	46.34	46.35
60	2.58 " " "	55.61	55.86
70	3.04 " " "	65.52	65.75
80	3.51 " " "	75.65	75.78
90	4.02 " " "	86.64	86.81
100	4.64 " " "	100.00	100.00

6.4 cc. $Ba(OH)_2$ × 79.15 per cent = 5.06 cc. $Ba(OH)_2$ for acetic acid.
6.4 " " " 5.00 " = 0.32 " " " propionic acid.
6.4 " " " 15.83 " = 1.01 " " " butyric acid.

*F = Formic acid; A = Acetic acid; P = Propionic acid; B = Butyric acid; V = Valerianic acid; C = Caproic acid.

Fraction II = 150 cc. and required 15.02 cc. $\frac{N}{10}$ Ba(OH)$_2$

DISTILLATE.	$Ba(OH)_2$.	FOUND.	CALCULATED FOR { A = 96.0, P = 2.5, B = 1.5 } Per cent.
cc.		*per cent.*	*per cent.*
10	0.63 cc. × $\frac{100}{8.18}$ =	7.70	7.67
20	1.28 " " "	15.65	15.69
30	1.95 " " "	23.84	24.00
40	2.65 " " "	32.40	32.77
50	3.40 " " "	41.56	41.74
60	4.18 " " "	51.10	51.33
70	5.03 " " "	61.49	61.37
80	5.96 " " "	72.86	72.53
90	6.97 " " "	85.20	84.80
100	8.18 " " "	100.00	100.00

15.02 cc. $Ba(OH)_2$ × 96.0 per cent = 14.42 cc. $Ba(OH)_2$ for acetic acid.
15.02 " " " 2.5 " " = 0.38 " " " propionic acid.
15.02 " " " 1.5 " " = 0.22 " " " butyric acid.

The total amount of volatile fatty acids in the 247.5 grams of whole milk cheese is calcuated from Fractions I and II. The acids are corrected to decinormal $Ba(OH)_2$.

	FRACT. I. $Ba(OH)_2$	FRACT. II. $Ba(OH)_2$	TOTAL.
	cc.	*cc.*	*cc.*
Acetic acid	5.06	14.42	19.48
Propionic acid	0.32	0.38	0.70
Butyric acid	1.01	0.22	1.23

In a similar manner the estimation and identification of the volatile fatty acids in the skim-milk cheese were carried out. Analyses of the curing cheese were made at definite intervals and the final data on this particular phase of the subject are brought together in the two following tables. All results are expressed as decinormal acid per 100 grams of dry matter.

TABLE I.

Whole Milk Cheese. In 100 grams of Dry Matter.

	3 DAYS OLD.	6 WEEKS OLD.	3 MOS. OLD.	5½ MOS. OLD.	10½ MOS. OLD.
Milk sugar........	.00	.00	.00	.00	.00
Lactic acid.......	84.09 cc. $\frac{N}{10}$	90.28 cc. $\frac{N}{10}$	124.00 cc. $\frac{N}{10}$	103.70 cc. $\frac{N}{10}$	74.10 cc. $\frac{N}{10}$
Total vol. acids ...	12.74 " "	34.17 " "	32.15 " "	34.82 " "	22.96 " "
Formic acid......	0.00 " "	0.00 " "	0.00 " "	1.80 " "	0.00 " "
Acetic acid.......	11.59 " "	29.47 " "	24.25 " "	25.86 " "	12.64 " "
Propionic acid....	0.41 " "	2.15 " "	3.42 " "	1.07 " "	2.63 " "
Butyric acid......	0.73 " "	2.17 " "	3.50 " "	4.82 " "	5.45 " "
Caproic acid......	0.00 " "	0.36 " "	0.96 " "	1.25 " "	2.23 " "
Succinic acid				20.60 " "	Trace

TABLE II.

Skim-milk Cheese. In 100 grams of Dry Matter.

	3 DAYS OLD.	6 WEEKS OLD.	3 MOS. OLD.	5½ MOS. OLD.	10½ MOS. OLD.
Milk sugar.......	.00	.00	.00	.00	.00
Fat	2.12 p. c.	3.06 p. c.			
Lactic acid......	149.27 cc. $\frac{N}{10}$	119.60 cc. $\frac{N}{10}$	179.44 cc. $\frac{N}{10}$	132.30 cc. $\frac{N}{10}$	11.60 cc. $\frac{N}{10}$
Total vol. acid ...	21.60 " "	62.29 " "	105.71 " "	84.20 " "	
Formic acid	0.00 " "	0.00 " "	0.00 " "	0.00 " "	
Acetic acid... ...	19.82 " "	46.13 " "	74.24 " "	57.10 " "	
Propionic acid....	1.48 " "	10.10 " "	10.51 " "	7.62 " "	
Butyric acid.....	0.29 " "	4.10 " "	17.42 " "	15.91 " "	
Caproic acid......	0.00 " "	1.94 " "	3.53 " "	3.56 " "	
Succinic acid				51.55	Trace

Discussion of the Data.

From the above tables it is apparent that the milk sugar disappeared early after manufacture, At the end of three days none could be detected.

Contrary to our working hypothesis, lactic acid did not disappear during the entire curing process. Its amount appeared to fluctuate somewhat, but in the whole milk cheese even at the end of 10 months, 88 per cent of that present at the time of the initial analysis still remained. In the case of the skim-milk cheese, during the later part of its history, lactic acid had largely disappeared.

The initial source of the lactic acid in the cheese is, of course, lactose; but the unmistakable increase in the amount of this body in both cheeses up to the end of three months and after all lactose had disappeared, raises the question of additional sources of this body during the curing process.

The supposition might be made that the lactose, during its fermentation yields, substances which later are slowly changed to lactic acid. It is a significant fact that the initial yield of lactic acid from the lactose in the curd is less than 50 per cent of the theoretical. One hundred grams of water-free cheese curd, one-day old, contain usually about 2 grams of lactose, which theoretically can yield 2 grams of lactic acid. Yet with what has been formed during the making process and up to the one-day stage, there was obtained but 0.75 gram.

In a lactose fermentation by mixed cultures of lactic acid organisms, besides the carbon dioxide, volatile fatty acids and lactic acid that are produced, such additional by-products as succinic acid and alcohol have been reported by several investigators. However, it is improbable that the latter substances are by further fermentation changed to lactic acid. It is more in agreement with the modern theory of lactic acid production, that the lactic acid itself is first formed, and by further breaking down, gives rise to simpler products. However, the occurrence of still unknown intermediary products capable of further change to lactic acid is not to be ignored.

A second possible source of lactic acid after the lactose fermentation lies in the proteolysis of the paracasein. Recht[1] has already reported the formation, during casein proteolysis by gastric juice, of such products as lactic and butyric acids. Even certain amino acids are theoretically possible precursors of this acid; as for example, serin, $CH_2OH.CHNH_2.COOH$, cystein, $CH_2SH.CHNH_2.COOH$ and alanin, $CH_3.CHNH_2.COOH$. The latter is particularly closely related to lactic acid and by simple deamidization and oxidation is converted to that body. Neuberg and Langstein[2] found that after administration of alanin to rabbits, small quantities of lactic acid appeared in the urine.

[1] *Compt. rend.*, lxxxvi, pp. 550–552; lxxxviii, pp. 750–751.

[2] *Maly's Jahresber. über d. Fortsch. d. Thierchemie*, p. 603, 1903.

Experimentally we were unable to induce such a change by inoculating a sterile 1 per cent solution of alanin with a small piece of whole milk cheese, 6 months old and about the size of a pea, and incubating at 35° C. for three months. Possible modifications and extensions of this experiment are in progress to further test such a theory.

The form of lactic acid in both whole and skim-milk cheese was, as previously stated, with but one exception, of an inactive variety. The skim-milk cheese when ten months old showed the presence of active lactic acid. 0.1392 gram of the crystalline zinc salt gave 0.0176 gram of water of crystallization, equivalent to 12.64 per cent. The theoretical requirement for the active acid is 12.89 per cent. Possible selective action on the part of some biological factor operative in this cheese in its later history would explain the above phenomena. This interesting observation that lactic acid might be of the active or inactive variety in cheese, dependent upon the nature of the biological factors inducing the fermentation, warranted more extended observations. For this purpose normal Cheddar cheese made at different seasons of the year and analyzed at different ages, as well as cheese made from milk, pasteurized at 160° F., was investigated for the form of lactic acid present. The results are appended in the following table.

TABLE III.

NO.	DATE MADE.	AGE.	REMARKS.	ZINC LACTATE.	WATER OF CRYST.	WATER OF CRYST
		mos.		*grams.*	*ams.*	*per cent.*
1	Mar. 1909......	1	Pasteurized	3.967	0.721	18.18
2	Sept. 1908......	9	"	0.939	0.173	18.42
3	" 1908......	9	"	0.570	0.105	18.42
4	Feb. 1909......	3	"	0.819	0.148	18.14
5	Aug. 1908......	10	Non-pasteurized	0.826	0.146	17.66
6	Feb. 1909......	3	"	0.470	0.085	18.17
7	Jan. 1908......	17	"	0.163	0.028	17.16

In all but two cases the agreement with the theoretical requirement for inactive lactic acid is close. Nos. 5 and 7 gave results lower than the theory required. Difference in the solubility of the zinc lactates made possible a separation, by fractional crystalli-

zation, of the active and inactive forms. The zinc salts of the active acids are more soluble than those of the racemic variety. This principle was applied to No. 5. From the first crystallization, 1.791 gram of the zinc salt gave 0.328 gram of water, equivalent to 18.31 per cent. From the second crystallization, 0.434 gram of zinc salt gave 0.062 gram of water, or 14.3 per cent. From the third fraction, 0.352 gram yielded 0.045 gram of water, or 12.76 per cent. The theory calls for 12.89 per cent for the active form. These results confirm the belief that there was present in cheese No. 5, active lactic acid, as well as the inactive form.

Source of the Forms of Lactic Acid.

In connection with this work on the forms of lactic acid in cheese, experiments were conducted to determine the variety of lactic acid produced in lactose solutions by various acid-producing organisms in the hope of throwing light on the source of the forms found in curing cheese.

Three hundred cubic centimeters of a lactose solution (1.8 per cent milk sugar, 0.5 per cent peptone and 5 grams of barium carbonate) were inoculated with a starter and incubated at 35° for two months. At the end of this time the lactic acid formed was examined. 0.216 gram of zinc lactate gave 0.0275 gram of water, equal to 12.73 per cent. Theory, 12.89 for the active salt. Three hundred cubic centimeters of the same solution, inoculated with a pure culture of *B. lactis acidi* and incubated at 35° C. for two months gave a similar result—always the active form of lactic acid.

The lactic fermentation of Cheddar cheese, so far as our investigation has gone, usually produced the inactive variety, a result in marked contrast to the above experiments with lactose solutions.

The explanation of this result may possibly be found in that (1) there is in cheese a more varied bacterial flora than is usually supposed. The results of numerous investigations indicate that 95 to 99 per cent of the bacteria in cheese belong to the *B. lactis acidi* group. It should be remembered that these results have been obtained by the use of methods that are favorable to the class of bacteria mentioned. Large numbers of other types of lactic organisms may be present. It is known that an organism similar to or identical with *B. Bulgaricus* is present in cheese in

unknown numbers. This organism has been shown by Heinemann to produce inactive lactic acid. (2) The active acid produced by *B. lactis acidi* is consumed by other organisms, while the inactive acid remains intact. The following experiment throws some light on the first supposition.

A milk sugar solution to which a small amount of fat had been added was inoculated with a bit of our whole milk experimental cheese. After four weeks' incubation at 35° the kind of lactic acid produced was investigated. Fractional crystallization of the zinc lactates gave the following results: 0.762 gram of the first fraction gave 0.138 gram of water of crystallization, equal to 18.12 per cent. Theory for inactive zinc lactate 18.18 per cent; 0.863 gram of the sixth fraction gave 0.110 gram of water of crystallization, equal to 12.78 per cent. Theory for the active zinc lactate 12.89 per cent. In the above experiments 1.76 gram of the zinc salt of inactive lactic acid, and 1.42 gram of the active acid were collected,

This experiment finds its simplest explanation in the supposition that unequal amounts of the active lactic acids are formed and that consequently several distinct lactose ferments were present in the solution. The inactive acid generally found in Cheddar cheese must very probably be referred to the formation of equal quantities of the two active acids by distinct ferments with the resultant racemic acid, or inactive form. To explain the paradoxical condition that equimolecular quantities of the *d*-acid and *l*-acid were formed by different ferments, it may be necessary to assume that the excess of one suffered destruction through the further action of other organisms. However, final conclusions cannot be drawn from the limited amount of data now at hand, and further investigations may show that small amounts of active lactic acid are present in normal cheese, and remain in the mother liquor after the separation of the less soluble zinc salt of the inactive variety.

Volatile Fatty Acids.

Whole milk cheese. The total amount of volatile fatty acids in 100 grams of dry matter of the whole milk cheese at the end of three days was equivalent to 12.74 cc. $\frac{N}{10}$ solution. This amount rose to 34.17 cc. at six weeks; remained at about that quantity to the end of 5½ months and then fell to 22.96 cc. at the age of ten months.

Skim-milk cheese. In the skim-milk cheese the amount of volatile acids is higher than in the whole milk cheese, probably due to the larger proportion of milk sugar in the curd, and was equivalent to 21.6 cc. $\frac{N}{10}$ acid at three days; this increased to 62.29 cc. at six weeks, 105.7 cc. at three months, and then decreased at the end of 5½ months to 84.2 cc. When we consider the individual acids, it will be seen that each had its own particular curve.

TABLE IV.

Acetic Acid.

(Results are expressed as cc. of decinormal acid.)

	3 DAYS.	6 WEEKS.	3 MONTHS.	5½ MONTHS.	10½ MONTHS.
Whole milk cheese........	11.59	29.44	24.25	25.86	12.63
Skim-milk cheese........	19.82	46.13	74.24	57.10	

In the whole milk cheese the maximum amount of this acid was found at the end of six weeks, and after that period it slowly decreased in amount until the end of 5½ months, after which a more rapid decrease took place.

In the skim-milk cheese there was a continual increase up to three months, after which the amount decreased.

These results in all probability only represent transition stages of the acid, and the combined result of constructive or destructive agencies. It is very probable that acetic acid is continually being formed during the cheese curing process and is also being destroyed, with the catabolic agencies more active in the later history of the cheese.

TABLE V.

Propionic Acid.

(Results are expressed as cc. of decinormal acid.)

	3 DAYS.	6 WEEKS.	3 MONTHS.	5½ MONTHS.	10½ MONTHS.
Whole milk cheese........	0.41	2.15	3.42	1.07	2.63
Skim-milk cheese........	1.48	10.10	10.51	7.62	

This acid behaved very similarly to acetic acid in that there was an increase during the early history of the cheese, with a gradual decrease as the cheese aged.

TABLE VI.

Butyric and Caproic Acids.

(Results are expressed as cc. of decinormal acid.)

	3 DAYS.	6 WEEKS.	3 MONTHS.	5½ MONTHS	10½ MONTHS
Whole milk cheese:					
Butyric acid	0.73	2.17	3.50	4.82	5.45
Caproic acid	0.00	0.36	0.96	1.25	2.23
Skim-milk cheese:					
Butyric acid	0.29	4.10	17.42	15.91	
Caproic acid	0.00	1.94	3.53	3.56	

On the whole, butyric and caproic acids increased in amount gradually during the entire curing process, but the amount of butyric acid was much higher than that of caproic acid. Again the quantity of both acids in the skim-milk cheese is very much larger than in the cheese made from whole milk.

Formic acid. This acid was obtained in the whole milk cheese at the age of 5½ months, but was not detected at any of the other stages in the analysis. At no time was it found in the skim-milk cheese.

It must be understood that it is not supposed that these acids existed in the free state in the cheese, but that they were either in loose combination with the nitrogenous substances of the curing mass, or as salts of the ash elements.

Source of the Volatile Fatty Acids.

Lactose as a source. The amount of volatile fatty acids formed during the period which marks the disappearance of lactose from the ripening cheese was unquestionable to be attributed to the lactose fermentation. The source of the increasing amounts formed after that time, is not as yet clearly defined. A number of experiments planned to throw some light on the sources of these acids are given in the following pages:

One hundred cubic centimeters of sterile lactose solution (1.8 per cent milk sugar, 0.5 per cent peptone, 5 grams of barium carbonate) when inoculated with either a pure culture of *B. lactis acidi* or a starter and incubated at 35° C., gave the following results at the end of three and fifty-six days, respectively:

	B. lactis acidi.		STARTER.	
	3 Days.	56 Days.	3 Days.	56 Days.
Formic acid........	0.43 cc. $\frac{N}{10}$	1.68 cc. $\frac{N}{10}$	0.57 cc. $\frac{N}{10}$	2.20 cc. $\frac{N}{10}$
Acetic acid........	3.88 " "	8.06 " "	16.15 " "	11.25 " "
Propionic acid.....	0.00 " "	0.00 " "	0.43 " "	0.13 " "

No propionic acid could be isolated from the *B. lactis acid.* culture; in addition no butyric or caproic acids were formed With the starter used, propionic acid was isolated, as well as formic and acetic, but neither butyric or caproic acids. The possibility that barium carbonate may have been somewhat toxic to the *B. lactis acidi*, thereby modifying the physiological processes of the organism, suggested itself. Consequently an experiment was made, using calcium carbonate as the neutralizing agent.

(*a*) 200 cc. solution of { 3.6 per cent milk sugar,
1 " " peptone,
2 " " calcium carbonate.

(*b*) 200 cc. solution of { 3.6 per cent sugar,
1 " " peptone,
7 " " barium carbonate.

They were inoculated with *B. lactis acidi* and, after incubation at 35° C. for two months, the solutions were analyzed, with the following results:

	(*a*)	(*b*)
Formic acid..............................	9.72 cc. $\frac{N}{10}$	4.92 cc. $\frac{N}{10}$
Acetic acid..............................	17.79 " "	1.44 " "
Propionic acid...........................	1.51 " "	2.12 " "

In this instance propionic acid was formed in both solutions, and it is also clear that the barium carbonate had exerted a

depressing influence on the metabolism of the organism. The kind of products formed was not affected. Neither caproic nor butyric acids could be isolated.

Experiments on lactose solutions with two distinct acid producing organisms, one a liquefying and the other a non-liquefying coccus, and both isolated from Cheddar cheese, gave the following results. The solutions used were identical with (*a*) as detailed above. The period of incubation was four months at 35° C. The results are given in the following table.

TABLE VII.

	LIQUEFYING COCCUS.	NON-LIQUEFYING COCCUS.
Acetic acid	28.6 cc. $\frac{N}{10}$	30.35 cc. $\frac{N}{10}$
Propionic acid	0.56 " "	1.07 " "
Butyric acid	0.70 " "	0.30 " "
Caproic acid	0.84 " "	0.20 " "

No formic acid was present in these solutions, but in both instances and even in the case of the non-liquefying coccus, caproic and butyric acids were formed. This is an important distinction from the products formed by the pure culture of *B. lactis acidi*, or the organisms contained in the starter used, which are largely, if not exclusively, *B. lactis acidi*. Not only was there a distinction in the character of the volatile fatty acids produced, but in addition it was impossible to separate lactic acid from the coccus fermentations.

The influence of one organism on another, whereby the character of their combined end-products is modified, finds an illustration in the following experiments. This experiment is presented at this point because of its important bearing upon interpreting results secured by fermentations with pure cultures and those in which a *number* of organisms have taken part.

Two hundred cubic centimeters of a lactose solution made as described above, and containing calcium carbonate, were incubated at 35° C. for periods of eight and fifty-six days respectively after inoculation with a mixture of *B. lactis acidi* and the yellow coccus. The results are shown below:

TABLE VIII.

	8 DAYS.	56 DAYS.
Formic acid	3.02 cc. $\frac{N}{10}$	5.08 cc. $\frac{N}{10}$
Acetic acid	6.86 " "	21.42 " "
Propionic acid	0.19 " "	1.33 " "

In pure culture the coccus had produced both caproic and butyric acids, but in the above experiment neither of these acids could be detected. The explanation of this result may be found in the more rapid growth of *B. lactis acidi* than the yellow coccus under the conditions obtaining in the experiment. The latter organism is very slow in its growth under all conditions, and is more susceptible to acid reaction in the medium. It seems probable that the growth of the coccus was very slight, and that the acids found were the result of the activity of *B. lactis acidi*. Further experiments are necessary to fully decide this point.

The fact that butyric acid was found in but small amounts during the period of direct lactose fermentation in the cheese, and caproic acid not at all, makes it seem probable that lactose was not the mother substance of these two acids, and further, that the organisms of the *B. lactis acidi* type were not responsible for the production of these two higher acids found in the curing cheese mass.

In another experiment 200 cc. of the lactose solution of the same composition as used in previous experiments were inoculated with a bit of the whole milk cheese, five months old, about the size of a pea, and incubated at 35° C. for 30 days. It was only possible to identify acetic and propionic acids as constituting the volatile acids formed during this fermentation. From the 200 cc. of lactose solution, the following quantities were obtained:

Acetic acid	348.5 cc. $\frac{N}{10}$
Propionic acid	174.0 " "

In an experiment, where 200 cc. of a solution of 1 per cent peptone containing 2 per cent of calcium carbonate but no lactose, were inoculated with a similar bit of cheese, as described above, and incubated at 35° C. for 4½ months, the following results were obtained:

Acetic acid	19.40 cc.	$\frac{N}{10}$
Propionic acid	4.47 "	"
Butyric acid	10.85 "	"
Valerianic acid	0.81 "	"
Caproic acid	3.08 "	"

Putrefaction had resulted, undoubtedly occasioned by the lack of an inhibiting effect on the growth of certain organisms, which would have resulted had lactose been present with the resultant production of high acidity. This experiment, however, makes it clear that certain volatile acids can have their origin in the decomposition of the protein molecule, a fact already well-known. A peptone solution, prepared as in the previous experiment, and inoculated with *B. lactis acidi* alone in pure culture, did not produce volatile fatty acids. This makes it very probable that that type of organism was not a factor in the formation of caproic and butyric acids in the cheese.

Lactates as Sources of Volatile Fatty Acids.

The possibility that the lactates found in cheese would, by further decomposition, yield fatty acids, has already been referred to in the early part of this paper. While lactic acid did not decrease during the normal ripening period, but in both instances actually increased, it may still have been in part the mother substance of volatile fatty acids. This increase, as previously suggested, may have had its origin in protein decomposition. Two hundred cubic centimeters of a calcium lactate solution (1.0 per cent calcium lactate, 0.5 peptone, 2 per cent calcium carbonate) were inoculated with a small bit of our whole milk cheese and incubated three weeks at 35° C. The separation and identification of the volatile fatty acids were made in the usual manner.

Acetic acid	8.70 cc.	$\frac{N}{10}$
Propionic acid	0.96 "	"

None of the other volatile fatty acids could be detected.

In another experiment of the same character and length of time, but inoculated with a bit of skim-milk cheese, the results were:

Acetic acid	10.08 cc.	$\frac{N}{10}$
Propionic acid	0.42 "	"

No putrefaction had occurred, as indicated by odor and the character of the acids. This makes it very probable that the lactates can act as the mother substance from which acetic and propionic acids may be formed by specific ferments[1] of the cheese.

It might be suggested that the above results are entirely due to enzymes contained in the cheese. The following experiment was planned to throw some light on this point. A milk sugar solution, containing peptone and calcium carbonate, as previously described, was inoculated with 2 grams of our experimental whole milk cheese, four months old, chloroform added to 2 per cent by volume, and the solution incubated 40 and 100 days respectively. No lactic acid could be isolated from the mixture while there was also a total absence of volatile fatty acids.

Even perfectly fresh milk preserved with chloroform and incubated one year at 35° C., failed to show the presence of lactic acid, indicating that there is no inherent lactose fermenting enzyme in cow's milk. A milk chloroformed and preserved at room temperature for eleven years still contained 5.06 per cent lactose and no lactic acid, confirming in every respect the above deduction.

Attempts to isolate an enzyme from curing cheese capable of attacking lactose, with production of volatile acids, also failed in every case.

Fats as a Source of Butyric and Caproic Acids.

All our experiments point to the conclusion that in the lactose or further lactate fermentation in cheese ripening, butyric acid is formed in but traces, and caproic acid not at all; nevertheless these acids increase steadily in amounts during the curing process. A possible source of these acids is caproin and butyrin, which are known to exist in milk fat. Either enzymes or organisms could be responsible for this hydroylsis. To determine whether or not this causal factor was contained in a starter, the following experiment was made:

Three hundred grams of fresh unsalted butter were washed several times with a large volume of hot water and then subjected to steam dis-

[1] The term "ferments" is here used collectively for both organisms and enzymes.

tillation until the distillate was neutral. Another portion of 200 grams of butter fat was acidified with dilute sulphuric acid and distilled with steam in order to determine whether or not the fats so treated in the usual method of volatile acid separation, would yield volatile fatty acids. The distillate of 1000 cc. required but 2.2 cc. of $\frac{N}{10}$ barium hydroxide. This is a negligible factor. Ten grams of the washed fat were used in the following experiment:

$$(a)\ 400\text{ cc. solution of}\left\{\begin{array}{lll}1.8 & \text{per cent} & \text{milk sugar,}\\ 0.5 & \text{"}\quad\text{"} & \text{peptone,}\\ 1.0 & \text{"}\quad\text{"} & \text{calcium carbonate,}\\ 2.5 & \text{"}\quad\text{"} & \text{fat.}\end{array}\right.$$

$$(b)\ 400\text{ cc. solution of}\left\{\begin{array}{lll}1.8 & \text{per cent} & \text{milk sugar,}\\ 0.5 & \text{"}\quad\text{"} & \text{peptone,}\\ 1.0 & \text{"}\quad\text{"} & \text{calcium carbonate,}\\ \text{No fat.} & & \end{array}\right.$$

Both solutions, after sterilization, were inoculated with a starter and incubated for 48 days at 35° C. The determination of the volatile fatty acids present, showed that only propionic and acetic acids had been produced in either solution, while neither butyric nor caproic acids could be identified in the distillates by the Duclaux method. This indicates that the starter contained no organisms or enzymes capable of lipolysis under the conditions of this experiment. The same experiment was repeated, but instead of inoculating with a starter, a bit of the experimental whole milk cheese, five months old, was used. At the end of a 30-day incubation, this solution was analyzed with the following results:

	Solution (a) plus fat.	Solution (b) no fat.
Acetic acid	138.52 cc. $\frac{N}{10}$	348.59 cc. $\frac{N}{10}$
Propionic acid	70.87 " "	174.00 " "
Butyric acid	38.29 " "	0.00 " "
Caproic acid	6.57 " "	0.00 " "

This experiment differentiates clearly the main source of the higher fatty acids and makes it probable that there are either active enzymes or organisms contained in cheese capable of hydrolyzing glycerides in the ripening process. Whether this action is due to lipases inherent in milk, or living organisms, must be decided by further experiments.

Proteins as a Source of Volatile Fatty Acids.

The fact that volatile fatty acids have been separated from protein decomposition, suggests the factor of proteolysis as an additional source of these products. In an experiment where

perfectly fresh whole milk was preserved for one year with chloroform, 100 cc. of the milk, equivalent to from 10 to 12 grams of solids, gave volatile acids equivalent in $\frac{N}{10}$ acid to 3.28 cc. Further work along this line is being conducted, in order to obtain sufficient material for identification of the volatile acids produced. Where a milk had been preserved for eleven years with chloroform, 400 cc., equivalent to from 40 to 50 grams of dry matter, contained volatile acids in quantity equivalent to 9.0 cc. of an $\frac{N}{10}$ solution. These acids on separation gave the following results:

Acetic acid	6.64 cc. $\frac{N}{10}$
Propionic acid	0.83 " "
Butyric acid	0.41 " "
Caproic acid	0.41 " "

It is, however, necessary to note that in an equivalent quantity of whole milk cheese, three months old, four times as much butyric and an equivalent quantity of caproic acid had been produced.

From our present data it is improbable that the combined action of lipolytic and proteolytic enzymes of milk are entirely responsible for the production of the higher fatty acids occurring in ripening Cheddar cheese. Further experiments, however, must fully decide this. That fatty acids are produced from protein decomposition alone by organisms or enzymes contained in the cheese, is shown by the following experiment:

Two hundred cubic centimeters of a solution of 1 per cent peptone and 2 per cent calcium carbonate, but containing no glycerides or milk sugar, were inoculated with a small bit of our whole milk cheese. After incubation for 135 days at 35° C. it was subjected to the Duclaux method with the following results:

Acetic acid	19.40 cc. $\frac{N}{10}$
Propionic acid	4.47 " "
Butyric acid	10.85 " "
Valerianic acid	0.81 " "
Caproic acid	3.08 " "

These results are in harmony with the well-known fact that extensive proteolysis gives rise to volatile fatty acid. It must

not be taken, however, as proof that this is a very important source of the volatile fatty acids produced during the cheese ripening process.

Glycerin as a Source of Certain Volatile Fatty Acids.

This body, which would be left after the hydrolysis of butyrin or caproin in the cheese mass, may by further fermentation give rise to acetic and propionic acids. This is shown in the following experiment:

Two hundred cubic centimeters of a solution of 0.9 per cent glycerin, 0.5 per cent protein and 2 per cent calcium carbonate, were treated with a small bit of the whole milk experimental cheese, and after incubation for 40 days at 35° C., gave the following results:

Acetic acid	21.8 cc. $\frac{N}{10}$
Propionic acid	1.17 " "

It is apparent that under the influence of the enzymes or organisms contained in the above cheese, glycerin could yield only acetic and propionic acids.

Isolation of Succinic Acid from Curing Cheese.

Succinic acid has been isolated from Emmenthaler cheese by Winterstein[1] and it has also been separated from solutions of lactose fermented by specific organisms;[2] it is probable that its origin in the cheese was from the early lactose fermentation.

However, the work of Ehrlich[3] on the formation of succinic acid from glutaminic acid makes it possible that protein decomposition can also be a source of this body.

In the process involving the separation of lactic acid from the three months old cheese, crystals appeared at that point in the process when the separated solution, neutralized with barium hydroxide, was evaporated to a small volume. They were

[1] *Zeitschr. f. physiol. Chem.*, xli, p. 485.

[2] Emmerling; *Zersetzung stickstofffreier organische Substanzen durch Bakterien*, p. 59.

[3] *Zeitsch. ver. Zuckerindustrie*, lix, p. 645.

observed in previous analyses, but regarded as crystals of barium lactate.

Separation. The mother liquor from the zinc lactate was treated with hydrogen sulphide to remove the zinc, then acidified with dilute sulphuric acid and extracted with ether. The ether extract was evaporated to a small volume, taken up with water and neutralized with barium hydroxide. When this solution was evaporated to a small volume, crystals began to separate. They were difficultly soluble in water, and brownish in color, due to impurities. They contained no water of crystallization, and on analysis showed 52.4 per cent of barium. Barium succinate contains 54.1 per cent, while barium lactate, with no water of crystallization, contains but 43.5 per cent. Barium citrate, which might have been present, contains 52.1 per cent of barium.

These results indicated that the body was either barium citrate or an impure barium succinate. Consequently, the filtrate and washings from the determination of barium were neutralized with barium hydroxide and silver nitrate added in excess. The precipitate was collected and dried at 108° C. 0.224 gram of this compound gave 0.144 gram of silver, equivalent to 64.3 per cent. The theoretical quantity of silver in silver succinate is 64.9 per cent, while for silver citrate the per cent is 63.1. This, we believe, identifies this body as a silver-succinate.

To further establish its identity the qualitative test used by Neuberg[1] was applied to the barium salt. This consists of heating in a test tube a portion of the salt with ammonia and zinc dust. When the excess of ammonia has been driven out a pine shaving, saturated with hydrochloric acid, is held at the mouth of the tube and the heating continued. A red coloration of the shaving shows the presence of succinic acid. With our preparation positive results were obtained.

The application of the test for citric acid described by Sabanin and Laskowsky,[2] failed to show its presence in this preparation.

This acid was again separated from both the whole and skim-milk cheese, at the 5½ months ripening stage. 1.192 gram of barium succinate from the whole milk cheese gave 0.175 gram of barium sulphate, equivalent to 53.6 per cent of barium. Theoretical quantity, 54.1 per cent. No analysis was made of that from the skim-milk cheese. This acid could not be directed in either the whole or skim-milk cheese at the age of 10½ months.

Succinic acid was also isolated from a solution of milk sugar and peptone, fermented in the presence of calcium carbonate, and fat, by inoculation with a small bit of the whole milk experimental cheese. No attempts were made to isolate this acid

[1] *Zeitschr. f. physiol. Chem.*, xxxi, p. 574, 1900.

[2] *Zeitschr. f. analyt. Chem.*, xvii, p. 74, 1878.

from the other experimentally fermented lactose solutions, but it is very probable that it had its origin in the cheese in the initial lactose fermentation of the curing curd.

"Flavor Solution."

As previously described, this solution was obtained from the first steam distillate by neutralization and redistillation. Neutral volatile substances, as alcohols and esters, would be found here, and it is a noteworthy fact that such solutions had a very strong flavor of cheese, as indicated by the sense of smell. Saponification and separation of the acids, as well as oxidation of the alcohols to acids and their separation, was carried on in a number of instances during these studies of the progressive changes in our experimental cheese.

A partial analysis was made of the solution obtained from both whole and skim-milk cheese, when six weeks old. Qualitative reactions for alcohols and aldehydes gave positive results for the former, but negative for the latter. Saponification and application of Duclaux' method established the presence of acetic, propionic and butyric acids in the solutions obtained from both the whole and skim-milk cheese. However, the amounts secured were small, necessarily making the separations less accurate than in the later analysis of the cheese. Oxidation of the alcohols was not carried out at this time.

Further studies of these solutions were made when the cheeses were three months old. Saponification and separation of the acids showed the presence of acetic, propionic, butyric and caproic acids. These had existed in the cheese as acid radicals in ester combinations. The same kinds of acid were isolated from the "flavor solution" obtained from the skim-milk cheese.

Oxidation of the Alcohols.

The distillate obtained from the saponified alkaline solutions was oxidized with potassium bichromate and sulphuric acid, as previously described, and according to the method of Dupré.[1]

The solution of acids obtained by oxidation of the alcohols

[1] *Journ. Chem. Soc.*, xx, 495.

from the whole milk cheese showed the presence of acetic, propionic and butyric acids. From the skim-milk cheese the same kinds of acid were obtained, after oxidation of the alcohols contained in its "flavor solution." The predominating acid formed in the oxidation of alcohols in the whole milk cheese, was acetic acid, with but small amounts of the two other acids present. These acids have originated from the oxidation of ethyl, propyl and butyl alcohols. Further the amount of acids obtained from the oxidation of the alcohols was much greater than that obtained by direct saponification. If saponification had been complete, and we believe it must have been, then a part of the alcohols must have existed in a free state in the cheese mass.

In the examination of the "flavor solution" obtained from both cheeses at the age of 5½ months, saponification and distillation gave acetic, butyric and caproic acids, but no propionic acid could be isolated from the whole milk cheese. From the skim-milk cheese the above four acids were isolated. In the case of the whole milk cheese, acetic acid again predominated, constituting 74 per cent of the acids isolated, while in the skim-milk cheese, which at that time was rather strong and pungent, butyric and caproic acids formed 85 per cent of the total acids isolated. Oxidation of the alcohols obtained from the saponified solutions yielded in both cases acetic, propionic and butyric acids. From the whole milk cheese 90 per cent of the acids obtained by this process were acetic, while but 4 and 6 per cent of propionic and butyric acids respectively were present. From the skim-milk cheese the same general proportions prevailed. Apparently ethyl alcohol was the chief alcohol occurring in the esters of the "flavor solution" obtained from either cheese.

Practically similar results were obtained from the analyses made at the age of 10⅓ months. These results, while by no means final and conclusive, strongly suggest the close relation of esters to the aroma of curing cheese, and which is detected by the sense of smell. The whole milk cheese, which was of good quality, had as a predominating component of its "flavor solution," ethyl alcohol and acetic acid, in all probability as esters; while in the skim-milk cheese of poorer quality, ethyl alcohol and caproic and butyric acids predominated as esters.

It is important for clearness that we distinguish between taste

and the aroma, the latter cognizant only through the sense of smell, and by which the quality of cheese is much *more* largely judged. In how far these esters characterize the taste and typify it for different varieties of cheese is, of course, impossible at present to state. In this connection, it is more than probable that the nitrogenous end-products also play an important part.

It is entirely probable that the alcohols had their origin, in part at least, in the primary lactose fermentation and further decomposition of lactates.

SUMMARY.

I. Lactose disappears from Cheddar cheese in from three to six days, dependent upon the condition of the milk and the temperature of curing.

II. The absolute amount of lactic acid found does not decrease during the ripening process, but may even increase. This increase after lactose disappears, is probably of protein origin.

III. The usual form of lactic acid found in Cheddar cheese is racemic. However, solutions of lactose, inoculated with *B. lactis acidi* or a starter, produced active acid, but when inoculated with a bit of cheese, a mixture of active and inactive acids was produced. Further work is necessary to explain this phenomenon.

IV. No enzyme capable of producing lactic acid or volatile fatty acids from lactose could be isolated from cheese. There is present in cheese a group of acid-forming organisms which produced no lactic acid in pure culture on a lactose solution, but did yield volatile fatty acids.

V. Volatile fatty acids were formed in increasing amounts during the ripening process, and after the lactose had disappeared. Acetic and propionic acids reached a maximum at three months and then decreased, while butyric and caproic acids continually increased during the experimental period covered. Formic acid was only detected in the whole milk cheese at the 5½ months stage. Valerianic acid was never obtained.

VI. The principal sources of acetic and propionic acids were probably lactates. Traces may have had their origin in protein decomposition, or further fermentation of glycerin.

VII. The principal sources of butyric and caproic acids were fats and proteins.

VIII. The distillate here designated "flavor solution" and characterized by the close resemblance of its odor to the cheese aroma, contained alcohols and esters.

IX. The "flavor solution" from the mild whole milk cheese contained esters made up largely of ethyl alcohol and acetic acid, while from the more pungent skim-milk cheese the esters were largely compounds of ethyl alcohol and caproic and butyric acids.

X. The alcohol probably had its origin in the lactose fermentation and appears to be an important factor in flavor production..

XI. The agencies operative in the production of volatile acids and synthesis of esters are as yet undefined.

XII. Succinic acid was isolated from curing Cheddar cheese and identified from its silver and barium salts.

ON A METHOD FOR THE DETERMINATION OF SODIUM IODIDE IN ANIMAL TISSUES.

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Owing to the vast amount of work that has been published in recent years on iodine-containing organs, there have coincidently arisen new methods for the determination of iodine. If one carefully scrutinizes the literature, it is apparent that really only one method has been used and subjected to minor modifications. This one basic method has been confined mainly to comparatively small quantities of iodine, such as the investigators have encountered in the thyroid gland, for instance. Where it is desirable to work with large quantities of iodine in the form of iodide, it will be readily realized that it is not only inconvenient, but also inaccurate to use the colorimetric method intended only for small quantities of iodine or iodide.

Rabourdin[1] originally devised the method used by Baumann and Ross,[2] Oswald,[3] Howald,[4] Anten,[5] Loeb,[6] Marine and Williams[7] and others.

The principal modifications proposed by Oswald and Anten were the use of the nickel crucible instead of the silver crucible by Oswald and the substitution of carbon disulphide for chloroform as a solvent for the liberated iodine by Anten. With respect

[1] Rabourdin: Liebig's *Annalen*, lxxvi, p. 375.
[2] Baumann and Roos: *Zeitschr. f. physiol. Chem.*, xxi, p. 481, 1896.
[3] Oswald: *ibid*, xxiii, p. 265, 1897.
[4] Howald: *ibid*, xxiii, p. 209, 1897.
[5] Anten: *Arch f. exp. Path. u. Pharm.*, xlviii, p. 331, 1902.
[6] Loeb: *ibid*, lvi, p. 320, 1907.
[7] Marine and Williams: *Archives of Internal Medicine*, May, 1908.

to an oxidizing and fusing capsule, the glazed porcelain dish is equally as serviceable as a nickel dish. The advantages of carbon disulphide, if any, are confined to the colorimetric method. The most recent method proposed for the determination of iodine is that of Hunter.[1] It is claimed that this method excels the various modifications of the original in "cleanliness, convenience, rapidity and accuracy." I have no experience with this method, but the methods heretofore used are not unduly complicated, nor exceedingly long when one becomes fairly accomplished in the actual usage of some one form or modification. Lack of detail furnished by the authors of the respective methods may perhaps account for objection to their usage in unskilled hands. It is the object of this paper to present briefly, but with sufficient explanation, a method not at all original, which has proven successful in our work.

The question of the selection of a method for the quantitative determination of sodium iodide arose in connection with a study of the absorption of this salt from the alimentary canal. The method had its inception in a former study on phenol absorption[2] but it has required further development in order to be utilized directly in connection with sodium iodide. Baumann found that the colorimetric method was inaccurate for quantities of iodine greater than 1.5 mgs. corresponding to 1.77 mgs. of sodium iodide, since the fine gradations between the colors of the chloroform solution were frequently missed; and in using large quantities of the thyroid gland, other organic snbstances or impurities gave the chloroform a peculiar tint which obscured or modified the violet color obtained with pure iodine. This experience of Baumann was repeated in our work. It was then proposed to use a volumetric method. In its entirety it consists of a combination of the fusion and oxidation method of Rabourdin with the titration of iodine in chloroform by sodium thiosulphate according to Fresenius.[3]

While Fresenius used carbon disulphide as a solvent for the iodine, chloroform was found to be equally satisfactory. Work-

[1] Hunter: *Proc. Soc. Exp. Biol. and Med.*, vii, No. 1, p. 10, 1909.

[2] Sollmann, Hanzlik, and Pilcher: *Journ. of Pharm. and Exp. Therap.*, i, no. 4, p. 409, 1910.

[3] Fresenius, R: *Traité d'analyse chimique quantitative*, 6th ed., p. 406, 1891.

ing with quantities 100 to 200 times as large as those of Baumann and others, and with organs larger; oftentimes with an unequal distribution of the sodium iodide; it was necessary to employ the whole organ for oxidation and fusion.

The procedure may be divided into three parts:

I. Desiccation; II. Fusion and Oxidation; III. Titration.

I. Desiccation. The organ and contents are carefully minced in a porcelain evaporating dish, by cutting the tissue into fine shreds, with scissors. Then the utensils are washed with a small quantity of distilled water into the minced mass. To the tissue are next added 3 to 5 cc. of 40 per cent sodium hydroxide and the whole is placed in a drying oven not exceeding 100° C. until the contents are dry. Sodium hydroxide is added to prevent the escape of any free iodine that may be liberated at this stage or during the later stages of fusion.

II. Fusion and Oxidation. When the contents of the dish are dry, it is placed over a gentle flame, the drying being continued until a black charred mass remains. Care must be taken that the flame does not rise to bring the mass to redness. At this point, considerable evolution of moisture, acrolein, carbonaceous matter and of malodorous fumes takes place so that the process is best conducted under a hood. When the contents are thoroughly dried and charred (usually requiring about two hours), a fusing mixture consisting of equal parts of sodium nitrate and sodium carbonate is added gradually with a spatula until all carbonaceous matter is oxidized, leaving a white residue. After the fused mass has cooled, it is moistened with a small quantity of hot distilled water by washing down the sides of the dish. It is then triturated and taken up with more hot water, the sides of the dish being thoroughly rubbed down with a rubber-tipped glass rod; the whole is filtered, and the filter washed with hot distilled water until no more iodide appears in the filtrate. The filtrate is then made up to a definite volume (250 cc. being a convenient quantity (and an aliquot portion (50 cc.) taken for titration.

III. Titration. To 50 cc. of filtrate in a separating funnel of 250 cc. capacity add gradually and with agitation 10 to 15 cc. of concentrated sulphuric acid. At this point a portion of the iodine will make its appearance giving the liquid a brown cast. Too much acid should not be used, since the heat of solution may

raise the temperature high enough to volatilize the liberated io.line; but the solution must be strongly acid to litmus paper. A small quantity (pinch) of crystalline sodium nitrite is added. This liberates the remainder of the iodine which is then shaken out repeatedly with 10 cc. portions of chloroform until the chloroform no longer acquires a violet tint. The chloroform solution is then washed with distilled water until it remains neutral and, finally, titrated directly with $\frac{N}{10}$ sodium thiosulphate until chloroform solution remains colorless.

One cubic centimeter of $\frac{N}{10}$ sodium thiosulphate is equivalent to 0.01498 gram of sodium iodide.

Several steps in the process require particular attention. First, oxidation must be conducted slowly and carefully so that it does not reach a bright red heat, since then there would be a loss of the iodide by volatilization. Second, one must always make certain with litmus paper that sufficient sulphuric acid has been added. Third, the chloroform-iodine solution must be washed with distilled water until it remains neutral, for if the solution should be acid then upon titration with sodium thiosulphate, iodine will be liberated from the sodium iodide A very small amount of acid in the chloroform-iodine solution was observed to bring about this disturbance. In such a case more sodium thiosulphate is utilized than actually necessary for the iodine contained and the iodine value will be too high.

Control Experiments.—In procuring data, portions of the alimentary tract of cats and dogs were used as the animal tissues. In each case loops of intestine of varying lengths and the stomach were ligated at opposite ends and the circulation from each severed in the dead animals. Then a solution of sodium iodide of a known strength in water was injected and allowed to stand different lengths of time ranging from ten minutes to two hours. At the end of their respective periods of time, the tissues were excised and carried through the analytical process. The results will be found in the following table:

Table of Results

EXP.	ANIMAL	ORGAN	SOJOURN	NO I INTRODUCED	NO I RECOVERED	LOSS— GAIN+	PERCENT RECOVERED	REMARKS
				Gram	*Gram*	*Gram*		
1	Cat	Loop of ileum 10 cm.	½ hour	0.0973	0.0936	0.0037−	96.19	
2	Cat	Loop of ileum 90 cm.	½ hour	0.0973	0.0973	0.0000±	100.00	
3	Cat	Whole stomach	10 min.	0.0973	0.0936	0.0037−	96.19	
4	Cat	Loop of ileum 10 cm.	10 min.	0.0247	0.0239	0.0008−	96.76	
5	Cat	Loop of ileum 10 cm.	½ hour	0.0247	0.0234	0.0013−	94.73	Loss of filtrate
6	Dog	Loop of ileum 90 cm.	½ hour	0.0973	0.0951	0.0022−	97.73	
7	Dog	Loop of ilium 10 cm.	2 hours	0.0973	0.0973	0.0000±	100.00	
8	Dog	Loop of ileum 10 cm	15 min.	0.1947	0.1962	0.0015+	100.76	
9	Cat	Loop of intestine 90 cm.	10 min.	0.2921	0.2846	0.0077—	97.43	
Average				0 1136			97.77	

DISCUSSION.

An average of 97.77 per cent of sodium iodide recovered from an average of 0.1136 gram used seems to justify the use of this method for quantitative work. In three instances 100 per cent was recovered. Why this could not be done in five other cases is not known, while in one instance there was a positive loss of the filtrate from the residue whence the low value of 94.73 per cent. It will also be noted that conditions as to time of exposure of iodide to the tissues varied. In an exposure of two hours (Experiment 7) there was recovered 100 per cent, the same being true of a thirty minute exposure (Experiment 2) and practically true for Experiment 8.

This method then is suited for the determination of large quantities of sodium iodide in animal tissues. The factor of unequal distribution of the iodide, complicated with the fusion and oxidation of large masses of tissue, does not seem to affect its recovery. The data for this method were obtained with quantities of sodium iodide 15 to 172 times greater than can be accurately determined by the Baumann method. It also incurs practically no loss of iodide which is bound to occur when portions of a composite sample for analysis are used. By this procedure comparatively large organs can be utilized, no portioning being necessary, therefore eliminating this error. It served with no loss in three instances, and only an average loss of 2.23 per cent in all instances when pieces of intestine ranging from 10 to 90 cms. in length were used.

Thanks are due to Prof. T. Sollmann for careful criticisms and suggestions on this work.

A CASE OF ALCAPTONURIA.

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Only five of the fifty-eight or so recorded cases of alcaptonuria have been reported as occurring in this country, namely, Brune's (1) case in 1886, Marshall's (2) in 1887, Ogden's (3) in 1894, Futcher's (4) in 1898 and the present case which was first called to our attention early in 1908. After examining seven samples of urine, practically the daily output for that number of days we were obliged suddenly to abandon further investigation for reasons given by one of us in the history of the case. We have delayed our report until the present time in the hope of resuming the examination. The chance that we can do so, however, is slight, and we have decided to publish such results as we have obtained.

Owing to the relatively small number of reported cases of alcaptonuria, this condition is regarded as rare. It probably is not common, though there is some reason for thinking it may be commoner than statistics would seem to show. Fromherz (5) has recently expressed the opinion that many alcaptonurics remain unnoticed and he has called attention to the fact that 45 out of 58 cases have been reported within the last fifteen years, or since Wolkow and Baumann (6) published their classical investigation on the subject.

There are at least two reasons why more cases of alcaptonuria have not been reported. In the first place, the weight of evidence thus far submitted is in favor of the view that alcaptonuria is without pathological significance. Because the condition is not pathological, the individual is at no discomfort and does not require medical care. Consequently the existence of alcaptonuria

has been discovered in many instances only indirectly when the individual has come under medical examination for some other cause. The individual in this investigation submitted herself to medical examination on account of alcaptonuria to be sure, but not because this condition had in any way interfered with her health. And this brings us to the second reason why so few cases of alcaptonuria have been reported, namely, the unfamiliarity of many physicians with the meaning of the word alcaptonuria and with the characteristic behavior of alcapton urine. A medical examination is not necessarily sufficient to disclose alcaptonuria, for life insurance examiners have rejected alcaptonurics thinking they were diabetics. Boedeker (7), who first described a case of alcaptonuria, was convinced that the urine in question contained sugar as well as another reducing substance responsible for the peculiar behavior of the urine and called by him alcapton. He thought sugar was present because of the persistent reducing power of the urine after removal of alcapton by basic lead acetate. As it is not clear that alcapton was completely precipitated, his proof of the presence of sugar is not conclusive. After two years Boedeker made a second examination of the urine of the same individual. He removed alcapton by Brücke's method and found that the urine gave a distinct reduction with bismuth reagent and an active fermentation in a few hours with yeast. Certainly these were reasons for suspecting sugar, yet Boedeker says it was not a genuine diabetes since the volume of urine in 24 hours was not unusual (1500 cc.) and the quantity of sugar was not over one per cent. It is possible that the individual examined was not a diabetic, for others since then have been deceived into supposing that alcaptonurics were diabetics. Brune (1), Marshall (2), Garnier and Voirin (8) and Futcher (4) were all called upon to re-examine the urine of individuals against whom had been recorded the diagnosis of diabetes with the result that they proved conclusively that alcaptonuria was the cause of the reducing properties of the urine. The behavior of alcapton urine, especially in the copper reduction tests, is so different from the behavior of urine containing sugar that it is difficult to understand how one could fail to be struck by the fact. It would seem as if doubt arising from the unusual reduction would lead to such confirmatory sugar tests as the fermentation and

polariscopic tests which would show that the substance, whatever it might be, could not possibly be sugar.

Garrod (9) who has given considerable attention to the subject of alcaptonuria has described this condition in one of his papers as follows:

> The urine has the normal color when fresh, but darkens from the surface downwards on standing, passing through various shades of brown to absolute blackness. The change follows much more rapidly when an alkali is added, *unde nomen alkapton*. One striking property of such urine is that of deeply staining any fabric which may be wetted with it, and the peculiarity is therefore readily noticed in infancy.
>
> Alcaptonuria is an extremely rare anomaly of proteid metabolism, usually congenital and life-long, and one which appears to be harmless. Of the recorded cases a number have occurred in brothers and sisters; only two instances of direct transmission from parent to child are known, and a large proportion of known alcaptonurics have been the offspring of consanguineous marriages. It is probably to be regarded as a "recessive character," in the sense in which that term is used by Mendel and his followers, latent in certain families, and more apt to appear when members of such families intermarry.
>
> As far as our knowlege goes, the alcaptonuric differs from the normal individual in that to him tyrosin and phenyl-alanin (products of proteid catabolism) do not undergo complete destruction in the ordinary way. The benzene ring of these compounds, which is normally broken up, passes unchanged through the alcaptonuric organism and appears in the urine in the form of homogentisic (hydroquinone acetic) acid, which is not improbably an intermediate product of normal metabolism. In a few cases a second aromatic acid (uroleucic) has also been found in the urine.
>
> The condition is recognized by the characters already described, and by the fact that the urine reduces Fehling's solution with the aid of heat and ammoniacal silver nitrate solution in the cold. Hence alcaptonurics are liable to be mistaken for diabetics.
>
> The urine does not yield Nylander's test for sugar, nor does it rotate the polarized ray. To recognize alcaptonurics with certainty, homogentisic acid must be extracted from the urine. It may be obtained without difficulty in the form of its lead salt, or as ethyl homogentisate. The former may be identified by estimating the water of crystallization or the contained lead, the latter by its melting-point and other properties.
>
> In some of the earlier cases the properties of the urine were ascribed to pyrocatechin, but no case of pyrocatechinuria simulating alcaptonuria has been described since the discovery of homogentisic acid by Wolkow and Baumann.

HISTORY OF THE CASE.

BY A. RAVOLD.

The circumstances which called my attention to this case of alcaptonuria are somewhat peculiar. Early in February, 1908, Mr. and Mrs. C. consulted me about their daughter who was engaged to be married. Before consenting to a marriage, the parents decided to get medical advice about an unusual condition which caused them some anxiety. They had noticed that their daughter's urine since her birth, though normal in appearance when first voided, gradually turned dark brown to deep black in color whenever it stood. If it soiled her undergarments, or came in contact with any kind of clothing, it left an indelible black stain. The mother produced several laundered articles which were either badly stained black in irregular areas, or spotted black in places. She was positive these stains were due to the urine and to nothing else. I also learned that she was equally sure this condition had existed since birth. Soon after birth she noticed that the child's soiled diapers became indelibly stained reddish-brown or black, depending on the length of time they stood before being washed. To prevent this staining, she had made it a practice to put all diapers or garments into a tub of water as soon as they were soiled.

I expressed a wish to examine this urine, and received the first sample on February 12, 1908, I still have a portion of this urine which is so black as to be entirely opaque when the small, flat container is held to the light. When first received, this urine was normal in every respect except in causing the appearance of a dark brown precipitate when added drop by drop to hot Fehling's reagent. Fearing this reagent had deteriorated in some way, though I kept the alkaline tartrate solution and the copper sulphate solution in separate bottles and mixed equal volumes of the two solutions as needed, I set the urine aside and had fresh solutions prepared for Fehling's reagent. On the following day I found a dark brown pigment-layer had formed on the surface of the urine to a depth of a quarter of an inch. Light brown flocculi were falling from this pigment-layer through the urine. I thought this pigment-layer might be due to a bacterial growth and asked for a second sample of urine which I received on the following day. This urine was also normal in every respect except that it gave the same result with Fehling's reagent. To determine whether the formation of pigment was due to bacterial action or not, I destroyed all bacteria by adding 25 cc. of formaldehyde solution and set the urine aside. It promptly behaved like the first sample. I was satisfied, therefore, that the change was not due to bacteria. I then had this urine thoroughly examined as described in another part of this paper.

The patient, aged 19, is 5 ft. 8 in. in height and weighs 150 pounds. Her chest measures 38 in. and her waist 26 in. She has blue eyes, blond hair and a fair complexion. She is of sanguine temperament. Her carriage is erect and alert. In every respect I should call her a perfectly healthy young woman. She has been under my observation practically

all her life. Except for an attack of measles at the age of seven, which lasted three weeks, she has always enjoyed good health. She has had no other illnesses and no accidents. Menstruation began at the age of fourteen. The flow has always been free, has lasted 3 to 5 days and has not been attended by pain or any abnormal symptoms.

That the urine might be thoroughly examined, the patient consented to collect it during the day and send it to the laboratory at the end of 24 hours. She did this for seven days. During the investigation she asked if anything had been found out as to the cause of her condition. I assured her it was nothing which would act detrimentally to her health as far as was known. Upon the strength of this information she took matters into her own hands and eloped, thus bringing further study of her condition to a sudden end.

Upon learning that the unusual behavior of this urine was due to homogentisic acid and that I was dealing with a case of alcaptonuria, I inquired into the family history of the patient, having been informed that several instances of alcaptonuria had been observed in individuals who were the offspring of consanguineous marriages. The patient is the elder of two children, the second child having died at birth. She is of German parentage on both sides and her genealogy is accurately known for four generations. There is no evidence of a consanguineous marriage having occurred in the family during that time. The father was born in Eschenau, Wurtemberg. He is a large, tall, well-proportioned man with blue eyes and blond hair. He is the fifth of twelve children. The mother was born in St. Louis. She is a small, well-formed brunette and the second of eleven children.

I also made inquiry regarding the patient's other secretions, since Stier (10) has shown the possibility of homogentisic acid being in cerumen, although his proof of such occurrence of this alcapton acid is not entirely conclusive. The mother was sure the sweat caused no staining, but could make no statement about the cerumen. But she thought the menstrual fluid produced stains independently of the urine.

Finally, I learned an interesting fact connected with the patient's trip abroad with her parents in 1907, at which time she visited her father's home in Eschenau. During a family reunion the father of the patient inquired of his male relatives and the mother of her female relatives if they had ever heard of black urine occurring in any member of the family. Nobody had ever heard of such a case in that family, but several of the women knew of a case of black urine in a young woman in their village. She was in no way related to their family, however. In that case the black urine had also existed from birth. This woman had married and after the birth of her first child the abnormality in the urine disappeared and did not return.

EXAMINATION OF THE URINE.

BY W. H. WARREN.

Physical Properties.

Appearance. When fresh, the urine was always bright and clear, though a slight cloudiness due to mucin appeared on standing. Other observers in speaking of the general appearance of alcapton urine have described it as perfectly clear and free from sediment.

Color. The urine was never quite fresh, since it was collected during the day, poured into a large bottle and brought to the laboratory on the following morning. The color under these conditions was not always the same. Yet there was never anything especially noteworthy about it. Four samples were distinctly reddish-brown and three pale yellow in color. Upon standing, the urine perceptibly darkened, and this change was the more pronounced the longer the urine stood.

The various statements regarding the color of alcapton urine differ. Several writers describe it as perfectly normal. But Fleischer (11) says it is first almost black, later brown by reflected and green by transmitted light and red on standing. Kirk (12) characterizes it as either pale, almost like water or a peculiar brown tint and never normal. Hirsch (13) says it is darker and browner than ordinary urine and that it is darker than normal urine on catheterization. I am inclined to regard some of these unusual colors as the result of the changes which set in when alcapton urine stands in contact with air, though of course this cannot apply to urine taken by catheter.

Odor. As regards this property I noticed nothing different from ordinary urine. Fleischer (11) has described the odor as sweet and aromatic and Futcher (4) as peculiar and slightly aromatic.

Volume. I am unable to say that the quantity of urine received represented in every instance the entire daily output. In fact, I learned that the 600 cc. sample was not the full amount. The volume on the other days varied from 930 cc. to 1425 cc., averaging 1050 cc.

From the statements of other writers, I am inclined to think that many alcaptonurics excrete either an excessive or a deficient quantity of urine. Yet, as some of these individuals had other maladies, perhaps these divergencies from the normal are of no particular significance as far as alcaptonuria is concerned. In the following instances the quantity of urine was less than 1000 cc., namely: Fürbringer (14), quantity small, average 600 cc.; Brune (1), 900 cc.; Ogden (3), 974 cc.; v. Moraczewski (15), 500--1200 cc. and later 200–400 cc.; Stier (10), 360–1030 cc.; Hirsch (13), 700 cc.; and Garrod (16), scanty and highly concentrated. On the other hand, the following writers have reported quantities exceeding 2000 cc., namely: Garnier and Voirin (8), 2000–4000 cc.; Stange (17), 1000-4000 cc. But more instances of deficient than of excessive quantity seem to have been recorded.

Specific Gravity. I determined specific gravity by hydrometer after having cooled the urine to 15° C. I obtained readings varying from 1.009 to 1.016 and averaging 1.013 which is about the same as the lowest readings of other observers.

The specific gravity of alcapton urine in several instances seems to have been high. Urines ranging from 1.020 to 1.025 have been recorded by Fürbringer (14) Armstrong (18), Smith (19), Brune (1), Futcher (4) and Noccioli and Domenici (20); and Hammarsten (21) and Schumm (22) have found some alcapton urines to run as high as 1.030.

Polarization. For examination in the polariscope I selected a sample of urine especially light in color. To make the color even lighter, I placed the urine in a small flask, from which air had been displaced by carbon dioxide, added purified boneblack and shook for some time. The urine was then filtered directly into a 10 cm. tube of a Schmidt and Haensch polariscope and examined in the usual way. The result was entirely negative.

Many other writers have applied this test to alcapton urines and without exception have obtained negative results. If possible, this test should always be made when there is any doubt as to the exact character of reducing substances in urine, especially if other properties such as are characteristic of alcapton urine have been observed.

Chemical Properties.

Acidity. I did not determine the acidity of the urine quantitatively, but merely tested each sample with litmus paper and found it decidedly acid. Alcapton urine seems to retain its acidity for a considerable time upon standing.

Zimper (23) titrated alcapton urine with $\frac{N}{10}$ sodium hydroxide solution and found that 100 cc. of urine required 1.8 to 4.3 cc. for neutralization and he had a urine containing from 0.7 to 1.27 per cent of homogentisic acid. Other observers have characterized the acidity as faintly acid, acid, distinctly acid and strongly acid, but always as acid.

Fermentation. Two tests were made side by side in Einhorn saccharimeters. In one tube I put alcapton urine mixed with fresh yeast and in the other tube two per cent glucose solution treated in the same way. The tubes stood for 12 hours in an air-closet at 37° C. In the test with glucose there was a considerable volume of gas in the tube but none in the test with alcapton urine.

Whenever this test has been applied to alcapton urine, the result has been negative. This test together with that with the polariscope should be made, if there is any doubt about the nature of the reducing substance in urine.

Reduction. Alcapton urine acts upon alkaline solutions of certain metals in a characteristic manner but these tests have been the means in some instances of leading to wrong conclusions regarding the cause of reduction. Yet these tests as given by alcapton urine and sugar need not be confused. For convenience, alkaline reduction tests as applied to alcapton urine may be divided into three classes.

(*a*) *Alkaline Copper Solutions.* Only upon application of heat will alcapton urine cause reduction in Trommer's and Fehling's tests, but to no extent in the cold. From the various descriptions of the copper reduction test as applied to alcapton urine, a distinct difference between it and the same test as given by sugar is evident. I think it would require a very careless observer to pass the test as given by our alcapton urine as a sugar test. I placed 10 cc. of Fehling's solution in a test-tube and added from a pipette 10 cc. of urine so that the latter rested on the surface

without mixing with the reagent. At the contact-zone an immediate black ring appeared, but no precipitate. Upon mixing the two liquids, I found that the blue color of the reagent entirely disappeared. The mixture was distinctly green and began to darken from the surface down. But there was no reduction in the cold. Even after placing the test-tube in a beaker of boiling water, I did not get an immediate reduction. The green color gradually disappeared and a yellowish precipitate of cuprous hydroxide formed. The supernatant fluid was deep black.

(*b*) *Ammoniacal Silver Nitrate Solution.* Alcapton urine immediately reduces this reagent in the cold. If the mixture is not agitated, silver will form a mirror on the test-tube, otherwise it will appear as a black precipitate. I found that our alcapton urine gave this test very beautifully. Wolkow and Baumann (6) have based the quantitative determination of homogentisic acid upon this reaction.

(*c*) *Alkaline Bismuth Solutions.* Alcapton urine will not reduce metallic bismuth as used in Nylander's and Boettger's tests, either cold or hot. I found that our alcapton urine gave a negative test with Nylander's solution. The mixture of urine and reagent begins at once to darken from the surface down and finally becomes black without the appearance, however, of the black precipitate formed when sugar solutions are similarly treated. No observer has reported this test otherwise than negative when applied to alcapton urine. For this reason it is especially valuable in differentiating an alcapton from a sugar urine and is much to be preferred to the copper test. Yet it is important to guard against mistaking the dark color due to the action of oxygen on the alkaline solution of homogentisic acid, for a black precipitate.

Phenyl-Hydrazine. I applied this test to alcapton urine and to two per cent glucose solution side by side. In one test-tube I put 10 cc. of urine and 5 cc. of freshly prepared aqueous solution of phenyl-hydrazine and glacial acetic acid; and in the second test-tube corresponding volumes of glucose solution and reagent. I heated the tubes in a beaker of boiling water. From the glucose solution I obtained the characteristic sheaf-like crystals of phenyl-glucosazone which I readily recognized under the microscope. But alcapton urine gave an entirely negative test.

Although the preceding examination shows sufficiently that the urine in question has all the characteristics of alcapton urine I thought it advisable after making these tests to supplement them with further experimental evidence, which I will now describe.

Isolation and Identification of Homogentisic Acid.

This alcapton acid was isolated from the urine by the method of Garrod (24).

About 150 grams of neutral lead acetate (6 grams for 100 cc. of urine) were dissolved in 2500 cc. of urine after it had been heated nearly to boiling. The greyish-yellow precipitate was collected at once on a Buchner filter and washed with a small quantity of distilled water. After standing 24 hours the filtrate gave a small additional deposit of crystals. The second precipitate was collected on a Buchner filter and washed as before. The combined precipitates, after being thoroughly air-dried, were powdered. This material weighed 8.35 grams. It was placed in a flask, covered with ether and decomposed by hydrogen sulphide gas. The ether solution was filtered from lead sulphide and then evaporated spontaneously. The residue consisted of prismatic crystals having a somewhat brownish yellow color and a distinctly urinous odor. Recrystallization from a small quantity of boiling water with the aid of bone-black gave a product which seemed quite pure.

These crystals, melted at 146–147° C., the melting point of homogentisic acid. An aqueous solution of the pure substance reduced hot Fehling's solution and cold ammoniacal silver nitrate solution, but did not reduce Nylander's solution. This solution made alkaline with potassium hydroxide solution began at once to darken from the surface down. By using a test-tube for this reaction, darkening was especially marked when the thumb was put over the mouth of the tube and the mixture vigorously shaken. The rapid absorption of oxygen caused a distinct pressure, an occurrence mentioned by several observers. Finally, dilute ferric chloride solution gave an evanescent blue color, a test which seems to be quite characteristic of homogentisic acid. There could be no doubt, therefore, that the substance in question was the particular aromatic acid responsible for the peculiar behavior of alcapton urine.

Quantitative Determination of Uric Acid and Homogentisic Acid.

These two acids were determined quantitatively in the same portion of urine, uric acid first being precipitated by Folin's (25) modification of Hopkins' (26) method, and homogentisic acid then being determined in the filtrate by the method of Wolkow and Baumann (6). In this way any reducing action which uric acid might have on alkaline silver nitrate solution was prevented in large measure.

Procedure. Filtered urine (100 cc.) was placed in a small Erlenmeyer flask with 15 grams of pure ammonium nitrate and just enough ammonia to make the solution slightly alkaline. The flask was tightly corked and the mixture allowed to stand over night. Alcapton urine when alkaline becomes darker and darker the longer it stands, especially if freely exposed to air. Under the conditions of the analysis by keeping the solution still there did not seem to be any extensive oxidation of homogentisic acid.

Ammonium urate soon began to separate and the precipitation was complete in the specified time. Usually it is sufficient in determining uric acid to let the solution stand two hours, but a longer time seemed to be advisable in presence of homogentisic acid. The solution after standing over night did not give a further deposit of ammonium urate.

The clear solution was decanted from the crystalline precipitate and filtered by means of the pump through a Gooch crucible having an asbestos mat. The filtrate was reserved for the determination of homogentisic acid by titration with $\frac{N}{10}$ ammoniacal silver nitrate solution. The crystals of ammonium urate adhering to the flask were washed with 10 cc. portions of 10 per cent ammonium nitrate solution. This wash-water was passed through the Gooch crucible and then added to the filtrate containing homogentisic acid. Two or three such washings were sufficient. The crystals and mat were then removed from the crucible and transferred to the flask containing the remaining crystals. Adhering crystals were finally washed from the crucible into the flask.

One hundred cc. of water and 15 cc. of concentrated sulphuric acid were added to the flask containing ammonium urate, the mixture was heated to 60° C. and titrated with $\frac{N}{20}$ potassium permanganate solution. Folin (25) has shown that 1 cc. of $\frac{N}{20}$ potassium permanganate solution represents 0.00376 gram of uric acid. The quantity of permanganate solution required to oxidize the uric acid in 100 cc. of urine varied from 3.5 cc. to 11.4 cc. and averaged 7.4 cc. The results are given in the table at the end.

In determining homogentisic acid, the filtrate from uric acid together with the wash water was made up to a volume of 150 cc. For each titration with $\frac{N}{10}$ silver nitrate solution 10 cc. of urine was mixed with 10 cc.

of 8 per cent ammonia recommended by Garrod and Hurtley (27). An immediate reduction of the silver solution took place. To determine whether too much or too little of the silver solution had been used, metallic silver was removed by filtration and the filtrate was tested first with $\frac{N}{10}$ silver nitrate solution and then with dilute hydrochloric acid after previous acidification with dilute nitric acid. Depending upon the result of these tests, a second titration was made with a smaller or larger volume of silver solution, and this was repeated until a filtrate was obtained which gave a precipitate neither with $\frac{N}{10}$ silver nitrate nor with hydrochloric acid. Usually four to six such tests were made. Wolkow and Baumann (6) have shown that 1 cc. of $\frac{N}{10}$ silver nitrate represents 0.0041 gram of homogentisic acid. The quantity of $\frac{N}{10}$ silver nitrate required to precipitate the homogentisic acid in 100 cc. of alcapton urine after removal of uric acid varied from 48 cc. to 120 cc. and averaged about 77 cc. The results are given in the table at the end.

The average daily quantity of uric acid in urine is usually placed at about 0.7 gram. I have not found a single sample of alcapton urine which I have examined to contain as much uric acid as that. To be sure, the first sample contained 0.514 grams, but in the six other samples the quantity was as small as 0.12 gram, an amount which must be regarded as abnormally low, and it never exceeded 0.3 gram. Apparently these results confirm the observation of several writers that the quantity of uric acid in alcapton urine may be deficient. Embden (28) found uric acid abnormally low (0.03 gram to 200 cc. of urine) as did Ogden (3). Fürbringer (14), Armstrong (18), Smith (19), Kirk (12), Stange (17) and Zimnicki (29) have also all called attention to an apparent deficiency, though it is not clear that their conclusions in every instance were based on quantitative determinations but rather on an apparent decrease as shown by the failure of uric acid to appear when alcapton urine is allowed to stand, a somewhat uncertain means of settling the question since we do not know what might have been the effect of the presence of homogentisic acid. But Zimper (23), who has found the largest quantity of homogentisic acid in alcapton urine, did not find a deficiency of uric acid. On the other hand, several writers have failed to notice any marked falling off in the quantity of uric acid in alcapton urine, among whom may be mentioned Schumm (22), 0.5 to 0.75 gram; Zimper (23), 0.59 gram; Hammarsten (21), 0.406 to 0.694 gram; and Garrod (16) who refers to this acid as not diminished.

Wolkow and Baumann (6) place the average daily amount of homogentisic acid at 4 grams. In a number of instances the quantity has been found to be less, Stier (10) having reported 2.6 grams; and both Embden (28) and E. Meyer (30), 3.2 grams. On the other hand, Schumm (22) found 7.5 grams; Langstein and E. Meyer (31), 6 to 8 grams; and Zimper (23) even 16.56 grams. In one sample I found slightly more than 4 grams of homogentisic acid. The average of the six remaining samples was 2.63 grams, in one of which the amount fell as low as 1.86 grams.

Quantitative Determination of Urea.

For the determination of urea I used a Doremus ureometer. In each case 1 cc. of urine was used and nitrogen was liberated by sodium hypobromite solution. Before reading the volume of nitrogen, the temperature of the gas was brought to 15° C. These results are only of comparative importance, however, since this method does not attempt to determine urea with the greatest accuracy. The results are given in the table at the end.

Too much dependence cannot be placed upon the total quantity of urea, for I am not sure that the volume of urine received always represented the total daily output. Although the method of analysis usually gives low results, the quantity of urea appears to have been somewhat below normal. Ogden (3) found the quantity of urea to vary as homogentisic acid varied. In a measure my results agree with this observation. Samples 1, 3 and 6 contained respectively 0.33, 0.35 and 0.41 per cent of homogentisic acid and they also gave the highest percentages of urea. On the other hand, samples 2 and 5 both gave 0.2 per cent of homogentisic acid, the lowest results, and they also gave the lowest percentages of urea.

Quantitative Determination of Total Nitrogen.

$$\frac{\textit{Homogentisic acid}}{\textit{Nitrogen}} \quad \textit{Quotient}$$

Total nitrogen in alcapton urine was determined by the Kjeldahl method in the usual way.

Results of Examination of Alcapton Urine

No.	Date April, 1908	Vol. in C.C.	Sp. Gr.	Urea: Total in grams	Urea: Per cent	Uric Acid: $\frac{N}{20}$ K Mn O_4 per 100 c.c. urine	Uric Acid: Total in grams	Uric Acid: Per cent	Homogentisic Acid: $\frac{N}{10}$ $AgNO_3$ per 10 c.c. urine	Homogentisic Acid: Total in grams	Homogentisic Acid: Per cent	Nitrogen: $\frac{N}{10}$ HCl per 5 c.c. urine	Nitrogen: Total in grams	Nitrogen: Per cent	H : N (N=100)
1........	9	1425	1,016	22.8	1.6	9.6	0,514	0,04	8.0	4.67	0.33	23.5	9.38	0.66	49.8
2........	10	1189	1,009	10.7	0.9	4.3	0,20	0,02	4.8	2.34	0.20	13.8	4.59	0.39	50.9
3........	11	600	1,015	9.0	1.5	11.35	0,26	0,04	8.5	2.09	0.35	24.1	4.05	0.68	51.6
4........	12	930	1,014	10.7	1.15	7.8	0,28	0,03	7.8	2.97	0.32	19.2	5.0	0.54	59.4
5........	13	945	1,010	8.5	0.9	3.5	0,12	0,01	4.8	1.86	0.20	14.1	3.73	0.39	49.9
6........	14	865	1,013	11.7	1.35	9.0	0,30	0,04	10.0	3.55	0.41	21.0	5.09	0.59	69.7
7........	15	965	1,012	11.1	1.15	6.2	0,23	0,03	6.0	2.37	0.25	18.5	5.0	0.52	47.4

Five cubic centimeters of urine were heated over a free flame with 15 cc. of concentrated sulphuric acid and oxidation of organic matter was hastened by a few crystals of pure copper sulphate. The residue when cold was diluted with water, neutralized with an excess of sodium hydroxide solution and distilled into $\frac{N}{10}$ HCl. Excess of acid was determined by titration with $\frac{N}{10}$ KOH, rosolic acid being used as an indicator. The results are given in the table at the end.

Though it was out of the question to attempt in any way to regulate the diet of the patient, since she lived at home and ate what she pleased, I have nevertheless calculated the $\frac{\text{homogentisic acid}}{\text{nitrogen}}$ quotient, N:H (N = 100), from the quantitative results obtained for these two products. If exception is made of the quotients 59.4 and 69.7, which may possibly be explained by the fact that No. 4 and No. 6 urines followed a diet containing a rather large quantity of milk, there is a remarkable constancy, since the results vary only between 47.4 and 51.6, averaging for these five samples 49.9. This average result is surprisingly close to the averages of similar determinations made by E. Mayer (30) Langstein and Falta (32), Schumm (22) and Garrod and Hele (33), as given by the last mentioned observers, namely:

	H		N
For the Basle case, Series *a*	49.8	:	100
" *b*	47.4	:	100
" *c*	49.9	:	100
" *d*	45.9	:	100
Schumm's case	49.8	:	100
Thomas P., Series *a*	45.8	:	100
" *b*	52.3	:	100
Albert P.	55.5	:	100
M.L.	44.9	:	100

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PHOSPHORUS IN BEEF ANIMALS[1]

Part I.

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Introduction.

This study constitutes but a part of the results obtained from the experiment *Uses to Which the Animal Puts its Food* now being conducted by the University of Missouri, and considers the quantity and to some extent the character of the phosphorus contained in the organs, tissues, etc., of beef animals.

The investigation is similar to that of Laws and Gilbert.[2] "A knowledge of the quantitative relation of the organs or parts, and of the ultimate and proximate composition of animal bodies, is of great interest in many points of view. Especially is a knowledge of the general composition of animals slaughtered as human food of great importance in the application of chemistry and physiology to dietetics. To the farmer, too, who is engaged in producing animal food for consumption—it is very desirable to know something of the chemical relations of the substances so produced and sold, to the constituents expended in producing it. In other words, he should possess some data for determining—what is the probable proportion of the consumed food, or of its several constituents, which he recovers in the form of meat? How much he may calculate as manure and how much as expenditure or loss by the fooding process."

[1]From the thesis presented to the Faculty of the University of Missouri by C. K. Francis in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2]*Phil. Trans.*, part 2, 1859; "Composition of Animals," Rothamsted Exp. Sta., 1858.

The investigators quoted above published their conclusions over half a century ago, and added another contribution later[1] but did not have access to modern apparatus or methods.

The most recent work in this same field has had for its chief object the perfection of analytical methods. Especially prominent in this respect have been the researches of Zaleski,[2] Hart and Andrews,[3] Koch and Woods,[4] Ivanhoff,[5] Schulze and Castoro,[6] and Emmett and Grindley.[7] Hart, McCullum and Fuller[8] working with pigs, have shown the rôle of inorganic phosphorus nutrition. Forbes[9] has demonstrated that the tenderloin of the pig contains 0.195 per cent to 0.352 per cent of phosphorus. Emmett and Grindley[10] claim that one-fourth of the total phosphorus in beef is in the soluble organic form and that different methods of cooking meat give products which differ as to the quantities and the nature of the phosphorus contents. The same investigators show the composition of several cuts from beef animals.

From unpublished data obtained in this laboratory it appears that raw meat contains fully 75 per cent of its total phosphorus in the organic form, and that the cooking of meat changes the organic phosphorus to an inorganic form depending in extent upon the temperature at which the meat is cooked. Proescher and Abderhalden [11] demonstrated the relationship between the composition of milk of different species and the time required to double the weight of their young according to the following table.[12]

[1] *Phil. Trans.*, 1883, p. 865.

[2] *Ber. bot. Ges.*, xx, p. 426, 1902.

[3] *Amer. Chem. Journ.*, xxx, p. 470, 1903.

[4] This *Journal*, i, nos. 2 and 3, 1906.

[5] *Ber. bot. Ges.*, xx, p. 366, 1902.

[6] *Zeitschr. f. physiol. Chem.*, xLi, p. 477, 1903.

[7] *Journ. Amer. Chem. Soc.*, xxviii, p. 25, 1906; *Journ. Ind. and Eng. Chem.*, i, nos. 7 and 8, 1909.

[8] *Amer. Journ. of Physiol.*, xxiii, p. 246, 1909.

[9] *Bull.* 81, Missouri Exp. Sta.

[10] *Journ. Amer. Chem. Soc.*, xxviii, p. 25, 1906; also *Ibid.*, xxvi, p. 1086, 1904; xxvii, p. 658, 1905.

[11] *Zeitschr. f. physiol. Chem.*, xxiv, p. 285.

[12] *Zeitschr. f. physiol. Chem.*, xxvii, p. 594.

TABLE I.

Species	Days from birth required to double weight	Per centage composition of milk			
		Protein	Ash	Calcium	Phosphorus
Man	180	1.6	0.2	0.021	0.022
Horse	60	2.0	0.4	0.086	0.057
Cow	47	3.5	0.7	0.114	0.087
Goat	22	3.7	0.78	0.143	0.122
Sheep	15	4.9	0.84	0.178	p.127
Swine	14	5.2	0.80	0.178	0.135
Cat	9.5	7.0	1.02		
Dog	9	7.4	1.33	0.321	0.223
Rabbit	6	10.4	2.50	0.636	0.437

This relationship is extremely interesting and important for it shows how essential to the development of the animal is the selection of appropriate food.

Characters of Animals Slaughtered.

All the animals except nos. 504, 523, and 525 were fed a mixture consisting of 2.5 parts grain (8 parts cracked corn to 1 part linseed meal) and 1 part alfalfa hay; the three mentioned received 2 parts grain to 1 part alfalfa hay, the grain consisting of 6 parts cracked corn, 3 parts whole oats and 1 part linseed meal. The general details of each animal are shown in Table II below.

An animal on maintenance is held at constant weight. The medium ration was designed to give the animal maximum thrifty growth without laying on fat. The sub-maintenance animal was made to lose one-half pound per day.

Method of Obtaining Samples.

At the time of slaughtering, which was done by an expert butcher, the weights of all organs were obtained after which they were grouped according to their functions for laboratory analysis.

Forty-eight hours later an expert from one of the large packing houses cut the right half of the carcass into the regular wholesale cuts. These were weighed and then separated into lean, fat and bone. In some cases the lean and fat of several cuts was grouped into composite samples for regular analysis and for the

TABLE II.

Character of Animals Slaughtered.

NO.	KIND OF ANIMAL	AGE	CONDITION	CLASSIFICATION OF CARCASS	REMARKS
18	Grade shorthorn steer	3 years 6 mos.	Very thin.	Cutter	On maintenance 6 months
43	Jersey cow	7 years 6 mos.	Fat	No. 3 beef	See note A. below.
48	Grade shorthorn steer	4 years 6 mos.	Very fat	No. 1 beef	Full feed for 21 months.
121	Grade shorthorn steer	3 years 6 mos.	Fairly fat	No. 1 beef	Full feed for 6 months.
504	Grade Hereford steer	1 year 9 mos.	Fat	Prime beef	Full feed all his life.
523	Grade Hereford steer	2 years	Medium	No. 3 beef	Medium ration all his life.
525	Grade Hereford steer	2 years	Thin	Good canner	Ration fed so as to cause ½ lb. gain per day all his life.
592	Grade Hereford steer	1 year 10 mos.	Emaciated	Poor canner	Submaintenance for 11 months. See note B. below.
594	Grade Hereford steer	11 mos.	Fat	No. 1 baby beef	Shorthorn Blood predominant. Full feed all his life.
595	Grade Hereford steer	1 year 9 mos.	Thin	Canner	Maintenance for a year.
597	Grade Hereford steer	1 year 6 mos.	Medium	No. 3 beef	Fed until fat, then held at maintenance for 7 motnhs.

NOTE A—Was kept on maintenance for a year, while giving milk, then dried up and held at the same weight until exact requirement was established. The feed was then changed to that mentioned above and the animal put upon full feed.

NOTE B—The condition of the skeleton of this steer was remarkable; the marrow having practically disappeared, being replaced with a watery malodorous liquid with none of the properties of normal marrow and totally lacking in greasy or fatty appearances.

determination of the water soluble portion, the composition of which is discussed in this paper.

Preparation of the Samples.

The cut or composite of cuts was first weighed in a tared container, then cut into small pieces and the entire sample passed twice through a meat grinder. First a coarse disc was used in the grinder then a finer one, the sample being completely mixed between each grinding. After quartering the sample was put through the grinder a third time. This process of grinding (with a gradual reduction of the coarseness of the disc used in the machine) mixing and quartering, was continued until the sample weighed about 1 kilo.

Details of Analytical Methods.

A weighing bottle was nearly filled, about 75 to 100 grams, with the well mixed sample, leaving room for a small aluminum spatula and the glass stopper. After weighing an approximate amount was transferred by means of the spatula to the proper vessel and the exact weight obtained by difference. All determinations were made in triplicate.

MOISTURE. The moisture content was determined on about 3 grams by the Benedict vacuum method as modified for this laboratory.[1]

FAT. The thimbles from the above determination were placed in Soxhlet extractors and extracted for twenty-four hours with ether distilled from sodium. The ether remaining in the thimble was driven off at a temperature not to exceed 60° C. and the tubes were then dried in vacuum dessicators as per above. The loss in weight represented the fat content. The results were very satisfactory, the triplicates generally agreeing closely.

ASH. About 10 grams of meat (15 grams if fat) were placed in a No. 0 porcelain crucible, heated for about two hours in an oven at 80°, then the temperature gradually raised to about 120° until thoroughly dried. When dried the sample was charred at a very gentle heat over a Bunsen burner. Very slowly the heat was increased to complete the combustion of the organic material. It was necessary to exercise considerable care to

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[1] P. F. Trowbridge: U. S. Dept. Agric. Bureau of Chem., Bull. 122, p. 215, 1908. L. F. Shackell: *Amer. Journ. of Physiol.*, xxiv, p. 325, 1909.

prevent fusion of the ash, frequently two days being consumed in the process. The residue, cooled, weighed and calculated to ash in the fresh sample.

TOTAL PHOSPHORUS. After the estimation of the ash was completed, each crucible was placed in a 250 cc. Jena beaker, sufficient nitric acid (sp. gr., 1.42) added to fill the crucible, then 10 cc. of hydrochloric acid (sp. gr., 1.21) together with a few cubic centimeters (5 to 10) of water, and heated on the water bath for two hours. Some samples needed six or eight hours digestion. The crucible was rinsed with hot distilled water and the contents of the beaker neutralized with ammonia (sp. gr., 0.90); a slight excess of nitric acid was added, then 100 cc. ammonium molybdate solution and the mixture heated to 65° in a water-bath for one hour. The solution was allowed to stand in a warm place for two hours; filtered (No. 597 S.&S. or no. 100 Swedish paper) and washed about five times alternately with a solution of ammonium nitrate which contained 100 grams in a liter,[1] and with cold water.

The original beaker containing traces of the yellow precipitate was placed under the funnel and the precipitate dissolved with dilute ammonia and hot water. Usually about six washings with 2.5 per cent ammonia were sufficient to dissolve the ammonium phosphomolybdate precipitate. The solution was then neutralized with hydrochloric acid and a few drops of ammonia added,[2] cooled, and 15 cc. magnesia mixture added slowly, with constant stirring. After a few minutes 15 to 20 cc. of ammonia (sp. gr., 0.90) were added and the solution allowed to stand at least two hours. Filtered and washed with 2.5 per cent ammonia solution, until free from chlorides: dried, ignited to whiteness and weighed as magnesium pyrophosphate.

PREPARATION OF THE SOLUTION FOR SOLUBLE PHOSPHORUS. Of the lean meats exactly 120 grams were weighed out in three portions, or 180 grams of fat samples in four weighings, and distributed into twenty 100 cc. Jena beakers[3] in approximately equal amounts. Fifty cc. of recently boiled, nitrogen-free water were measured out and the portion of meat in beaker no. 1 moistened with about 5 cc., then mixed with a stirring rod to a pasty, condition; more water added and mixed until the whole 50 cc. had been added. This operation was repeated with each of the 20 beakers. After standing about one-half hour with frequent stir-

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[1] Satisfactory results have been obtained by alternate washings of the filters with ammonium nitrate solution and water.

[2] W. Pawlenko (*Vyestink Sakh. Promnish.*, No. 37, p. 417, 1906) finds that alkaline magnesia mixture gives as accurate results as the neutral mixture (Abs., *Exp. Sta. Record*, xx, p. 111, 1908).

[3] The beakers were numbered and the division of the different portions indicated, so that if a beaker was broken it was not necessary to reweigh the whole sample, but only that portion from which the loss occurred.

ring, the extract was poured onto 11 cm. (No. 595 S.&S.) filters and filtered into 300 cc. Florence flasks without permitting the major portions of the residue to flow from the beakers. If during the process, any considerable amount of the meat residue collected upon the filter, it was returned to the corresponding beaker with the aid of the stirring rod. Next 25 cc. of the neutral, nitrogen-free water was added to the residue in each beaker, mixed thoroughly and poured on the filter when the first portion of the extract had all passed through. This was repeated until eight 25 cc. portions of the water had been used in addition to the first 50 cc. portion, making 250 cc. of extract from each portion. The residue in the beaker was transferred to the filter with the last 25 cc. of water, the beaker and filter washed twice with 10 cc. portions of water. This made a total of 270 cc. of water used for each flask.

The extract was transferred to a 2-liter measuring flask and then to a glass stoppered bottle of approximately 8 liters capacity. Each Florence flask was rinsed twice with about 12 cc. of water. The 6 liters[1] of the extract were mixed, avoiding aeration, and filtered through a dry filter.

Total Soluble Phosphorus. 500 cc. portions of the extract were measured into 600 cc. beakers and evaporated on the water bath to a volume of about 50 cc; with the aid of 15 cc. of sulphuric acid and hot water this was transferred to 500 cc. Kjeldahl flasks. Then about 0.7 gram mercury together with 5 grams of potassium sulphate were added and the solution digested as for nitrogen determinations.

After cooling, the liquid and any residue were washed into a 250 cc. beaker, slightly diluted with water and neutralized with ammonia. The operation was then completed as under total phosphorus.

Soluble Organic Phosphorus.[2] 600 cc. portions of the extract were measured into 1000 cc. Erlenmeyer flasks, 5 cc. of 10 per cent barium chloride solution, 10 cc. of ammonia (diluted, 1:1) and 45 cc. of water added making a total of 660 cc. representing 12 grams of the original sample; thoroughly mixed, covered with a watch glass and allowed to stand over night or until the precipitate had settled. Filtered through a dry filter and 605 cc. (eleven-twelfths) of the filtrate,[3] placed in a dry 1000 cc. Erlenmeyer flask, 10 cc. of 5 per cent potassium sulphate solution and 45 cc. of water added, making a total volume of 660 cc. Thoroughly mixed and allowed to stand long enough for the precipitate to settle, then filtered or decanted, according to the condition of the precipitate. 600 cc. (five-sixths of the original sample) were measured into 800 cc. beakers and treated in the same manner as under total soluble phosphorus above.

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[1]Excess of solution was used for other determinations than those mentioned below.

[2]Siegfried and Singewald: *Zeitschr. f. Nahr. Genussm.*, x, p. 52, 1905.

[3]Representing 11 grams of the sample of lean meat or 16.5 grams of fat meat.

Soluble Inorganic Phosphorus. The difference between the organic and the total soluble phosphorus was considered to be inorganic phosphorus.

Originally attempt was made to ascertain the amount of this constituent by means of the Hart and Andrews method[1] as modified by Emmett and Grindley[2] but the results were not satisfactory. The method was used on a considerable number of samples, the results of which compared favorably with those of Emmett and Grindley, showing practically all of the phosphorus present to be in the inorganic form. However, it became apparent that the percentage of organic phosphorus, when obtained by differences, did not correspond with actual organic phosphorus according to the method of Siegfried and Singewald.[3] It was thought that an error was introduced through the fact that in the Emmett and Grindley method, and all others met with in the literature, the solution was heated before precipitation. Data[4] obtained in this laboratory from experiments on the cooking of meats seemed to warrant the above assumption. These experiments, as previously mentioned, indicated that the inorganic phosphorus was increased through the cooking. In order to study this question and compare the methods a series of experiments were undertaken.

Experiments with Water Solutions of Beef.

The solutions were prepared by mixing 600 grams of the lean meat with about 1000 cc. of water and pouring on a cheese cloth filter. After squeezing, the residue was returned to the mixing vessel (a thick glass jar), another 1000 cc. portion of water added and after mixing again poured on the filter. This operation was repeated six or seven times. The volume of the liquid was made up to 15 liters and then divided into three parts (*a*, *b* and *c*) of 5 liters each and each portion filtered through a dry filter (18 cm. S. & S. No. 595).

Part *a* was analyzed as follows:

(1) Three portions of 500 cc. each marked *G(H&A)* for inorganic phosphorus according to Emmett and Grindley's[5] modification of Hart and Andrew's method.

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[1]Hart and Andrews: *loc. cit.*

[2]Emmett and Grindley: *loc. cit.*

[3]Siegfried and Singewald: *loc. cit.*

[4]*Journ. of Ind. and Eng. Chem.*, ii, May, 1910.

[5]*Journ. Amer. Chem. Soc.*, xxviii, p. 25, 1906.

(2) Three portions of 500 cc. each, marked *g*, *h*, *i*, for total soluble phosphorus by the method described above.

(3) Three portions of 600 cc. each marked *j*, *k*, *l*, for organic phosphorus by Siegfried and Singewald's method already mentioned.

Part *b* was placed in a large Florence flask of about 6.5 liters capacity, closed with a rubber stopper fitted with a reflux condenser and from which a thermometer was suspended in the liquid. The flask was then immersed in a deep water bath and stirred by shaking occasionally while the temperature was gradually raised to about 60° and maintained for 15 minutes. The temperature of the liquid and the bath were recorded every five minutes and at no time were the two readings over a few degrees apart. After cooling, usually overnight, the solution was filtered and then analyzed by the same methods mentioned for part *a*.

Part *c* was also heated under the same conditions, but the temperature in each case was somewhat higher than that employed for part *b*. The time during which the maximum temperature was maintained was the same.

The results of these experiments are set down in Table III.

Discussion of Data.

The results given include the data from the preliminary tests (Series 993) and while the figures are not as uniform as those obtained from later experiments, when the details had been perfected, the general trend is to be observed.

Attention should be called to the fact that part *a* 91118, was prepared according to the regular water extract method, as described on page 486, while parts *b* and *c* were made by weighing another portion of the same sample and extracting by the special method adopted for these tests. The total soluble phosphorus determinations indicate the efficiency of the special method, at least so far as the phosphorus content is involved.

Since in the regular analysis of the cold water extracts only the Siegfried and Singewald method was used, the determinations by the Emmett and Grindley method are lacking for part *a*. In the same series parts *d* and *c*, heated to 70.3° and 90.7° respectively, the inorganic phosphorus determined according to Emmett and Grindley shows considerably lower results than with any of the other experiments; this discrepancy cannot be explained.

A comparison of the total phosphorus in the cold solution with that obtained after removing the coagulum formed by heating at the different temperatures, indicates that practically no phos-

TABLE III

Effect of Heat upon the Form of Phosphorus in Meat Solutions.

NO.	SOURCE OF SAMPLE	MAXIMUM TEMPERATURE	PHOSPHORUS (PER CENTS)			
			Total	Organic	Inorganic by difference	Inorganic by G. (H. & A.)
993*a*.......	Round lean.........	Cold (20°)	0.164			0.147
			0.165	0.106	0.059	0.142
			0.162	0.067	0.095	0.146
b......		60.2°	0.164	0.014	0.150	0.145
			0.164	0.013	0.151	0.139
			0.164	0.012	0.152	0.131
c......		70.2°	0.146	0.012	0.134	0.125
			0.143	0.012	0.131	0.126
			0.144	0.017	0.127	0.129
91118*a*.......	Chuck and neck steer no. 523......	Cold (20°)	0.131	0.086	0.045	
			0.130	0.086	0.044	
			0.127	0.082	0.045	
b......		70.3°	0.126	0.024	0.102	0.094
			0.126	0.022	0.104	0.079
			0.126	0.023	0.103	0.077
c.......		90.7°	0.125	0.016	0.109	0.070
			0.125	0.022	0.103	0.079
			0.127			
9928*a*.......	Round lean.........	Cold (20°)	0.136	0.070	0.066	0.132
			0.146	0.104	0.042	0.125
			0.146	0.092	0.054	0.129
b......		50°	0.147	0.067	0.080	0.126
			0.147	0.066	0.081	0.131
			0.146			0.133
c......		70.5°	0.144	0.024	0.120	0.125
			0.146	0.025	0.121	0.130
			0.146	0.024	0.122	0.127
91027*a*.......	Lean composite from cow no. 4..	Cold (20°)	0.118	0.062	0.056	0.118
			0.137	0.080	0.057	0.118
				0.083		0.117
b......		51.7°.......	0.098	0.025	0.073	0.118
			0.139	0.033	0.106	0.119
			0.111			0.117
c......		70.2		0.027		0.120
			0.139	0.028	0.111	0.118
			0.139	0.028	0.111	0.118

phorus is precipitated with the coagulum. This conclusion agrees with that of Emmett and Grindley.[1]

A portion of the organic phosphorus is converted into the inorganic form when heated above 50° and therefore the method proposed by Emmett and Grindley, or any method which involves heating of the cold water extract before precipitation of the inorganic phosphorus, cannot represent the true condition of the phosphorus in the flesh.

The relation of the inorganic and organic phosphorus to the total phosphorus as shown in the above table, may be seen in Table IV in which the data have been averaged and calculated to per cents in each case.

In the samples examined it appears that the cold extracts contained from 52 to 65 per cent of the total soluble phosphorus in the organic form, which when heated to about 70°, was reduced to from 9 to 20 per cent, accompanied by a corresponding increase of the inorganic phosphorus. The change occurred to a greater or less degree at other temperatures, but seemed to be practically complete at 70° as is especially shown in Series 91118. Here it will be observed that there was only 3 per cent less organic phosphorus in the solution after heating to 90.7° than was found in part *b* which had been heated to 70.3°.

Water Extracts of Beef.

The data presented in this part were obtained from the analyses of selected cuts from steers 504, 523 and 525. The history of these animals has been given on page 484; they were in the order named above, fat, medium and thin respectively. For purposes of comparison and to help interpret the results, water and fat determinations made on the original sample are included in the tables. All analytical data are expressed in per cents.

From the data reported in Table V it is evident that the round lean contains more phosphorus, in forms which are soluble in cold water, than any of the other cuts; the lean of the loin con-

[1] *Journ. Amer. Chem. Soc.*, xxviii, p. 1906.

TABLE IV.

Percentage Relations of Organic and Inorganic Phosphorus to Total Phosphorus in Meat Solutions.

Number	993.			91118.			9928.			91027.		
Part	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
Temperature	Cold	60.2°	70.2°	Cold	70.3°	90.7°	Cold	50.0°	70.5°	Cold	51.7°	70.2°
Total phosphorus	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Organic phosphorus	52.76	7.93	9.44	65.73	18.25	15.20	62.23	45.23	16.72	58.82	25.00	20.14
Inorganic phosphorus by difference	47.24	92.07	90.56	34.27	81.75	84.80	37.77	54.77	83.28	41.18	75.00	79.86
Inorganic phosphorus by Emmett and Grindley	88.41	84.14	88.19		65.71	57.14	90.20	88.43	86.98	92.12	101.7	85.61

TABLE V.

Partial Composition of Selected Cuts from Steer No. 504.

NUMBER	DESCRIPTION OF SAMPLE	TOTAL SOLUBLE SOLIDS	WATER	FAT	PHOSPHORUS		SOLUBLE PHOSPHORUS				
					Total	Insoluble	Total	Total water- and fat-free	Total fat-free	In-organic	Organic
81220	Lean and fat of shin, shank, head and tail	3.44	60.64	20.56	0.143	0.045	0.098	0.710	0.123	0.089	0.010
81221	Lean and fat of chucks and neck	3.69	58.33	24.03	0.146	0.046	0.100	0.567	0.131	0.131	0.017
81222	Lean and fat of flanks and plates	2.27	41.64	45.62	0.101	0.032	0.069	0.537	0.126	0.029	0.041
81223	Lean and fat of rumps	2.55	40.72	46.81	0.106	0.029	0.077	0.617	0.145	0.031	0.046
81224	Lean of rounds	5.98	69.51	9.21	0.194	0.013	0.181	0.850	0.199	0.093	0.088
81225	Fat of rounds	0.57	16.61	78.03	0.030	0.019	0.011	0.205	0.050	0.011	0.000
81226	Lean of loins	5.16	66.92	12.22	0.181	0.058	0.123	0.589	0.140	0.073	0.050
81227	Fat of loins	0.43	11.62	84.91	0.025	0.010	0.015	0.432	0.099	0.014	0.013
81228	Lean of ribs	4.63	63.28	17.52	0.167	0.039	0.128	0.666	0.155	0.088	0.040
81229	Fat of ribs	0.75	14.42	80.63	0.031	0.017	0.014	0.282	0.072	0.015	0.000

taining the smallest amount. The following table shows the quantity of the total phosphorus which is soluble.

TABLE VI.

Cuts of Steer No. 504 Arranged According to Percentage of Total Phosphorus which is Soluble.

DESCRIPTION OF SAMPLE	TOTAL PHOSPHORUS WHICH IS SOLUBLE
Round lean	93.3
Rib lean	76.6
Rump	72.6
Shin, shank, head and tail	68.5
Chuck and neck	68.4
Flank and plate	68.3
Loin lean	67.9
Loin fat	60.0
Rib fat	45.1
Round fat	36.6

The soluble inorganic phosphorus ranges from 0.011 to 0.015 per cent in the fats examined; from 0.029 to 0.089 in those cuts in which the lean and fat was combined; from 0.073 to 0.093 in the lean cuts.

Table V seems to indicate that the lean round, a medium price cut, contained practically the same amount of total phosphorus as the lean loin, an expensive cut. Moreover, the amount of soluble phosphorus was considerably less in the lean loin.

To aid in the study of these relations the data were arranged in the order of the total soluble phosphorus content as shown in Table VII.

It appears from the above tables dealing with steer no. 504 that the fats contained very little soluble phosphorus, an average of 0.013 per cent; the cuts in which the lean and fat were combined for analysis contained from 0.07 to 0.10 per cent; the lean cuts from 0.12 to 0.18 per cent. When the results are reduced to a dry and fat-free condition the percentage of total soluble phosphorus in the fats becomes 0.2 to 0.43 per cent; lean meat referred to the same basis contains from 0.59 to 0.85 per cent. When the meat is freed from both water and fat the results are raised considerably; the fats from 0.20 to 0.43; the lean cuts from 0.53 to 0.85.

TABLE VII

Cuts of Steer No. 504 Arranged According to Percentage of Total Soluble Phosphorus Referred to the following Conditions of the Cuts.

DESCRIPTION OF SAMPLE	FRESH	WATER- AND FAT-FREE	FAT-FREE	WEIGHT OF CUTS
				Grams
Fat of rounds..................	0.011	0.205	0.050	9818
Fat of ribs......................	0.014	0.282	0.072	6770
Fat of loins.....................	0.015	0.432	0.099	18340
Lean and fat of flanks and plates	0.069	0.537	0.126	49650
Lean and fat of rumps..........	0.077	0.617	0.145	10846
Lean and fat of shins, shanks, head and tail...............	0.098	0.710	0.123	16070
Lean and fat of chucks and neck	0.100	0.567	0.131	59808
Lean of loins....................	0.123	0.589	0.140	33676
Lean of ribs.....................	0.128	0.666	0.155	18506
Lean of rounds..................	0.181	0.850	0.199	37238
Composite*......................	0.076	0.543	0.127	76566

* The results shown here were obtained by calculation from the data reported on the fat and lean of the following: shin, shank, head and tail; flank and plate; rump.

TABLE VIII

Partial Composition of Selected Cuts from Steer No. 523.

LAB. NUMBER	DESCRIPTION OF SAMPLE	TOTAL SOLUBLE SOLIDS	WATER	FAT	SOLUBLE PHOSPHORUS Total	SOLUBLE PHOSPHORUS Total Water and Fat-Free	SOLUBLE PHOSPHORUS Total Fat-Free	SOLUBLE PHOSPHORUS Inorganic	SOLUBLE PHOSPHORUS Organic
91118	Lean and fat of chuck and neck..............	4.83	72.78	10.29	0.129	0.762	0.144	0.043	0.086
91121	Lean of round	5.59	78.26	1.93	0.154	0.777	0.157	0.029	0.125
91122	Fat of round...........	1.03	35.63	60.22	0.027	0.190	0.068	0.015	0.012
91123	Lean of loin............	5.82	74.07	10 41	0.152	0.979	0.169	0.053	0.099
91124	Fat of loin.............	0.82	16.50	77.94	0.016	0.286	0.073	0.005	0.011
91125	Lean of rib.............	4.59	75.40	9.30	0.139	0.908	0.153	0.067	0.072
91126	Fat of rib..............	1.49	24.11	64.98	0.029	0.266	0.083	0.013	0.016
91136	Composite of leans and fats exclusive of above samples*.............	3.23	63.80	20.41	0.105	0.664	0.132	0.029	0.076

* This composite sample was made by combining aliquot parts of the lean and fat of the following: shin, shank, head and tail; flank and plate; rump.

TABLE IX.

Cuts of Steer No. 523 Arranged According to Percentage of Total Soluble Phosphorus. Referred to Different Conditions of the Cuts.

DESCRIPTION OF SAMPLE	FRESH CONDITION	WATER AND FAT-FREE CONDITION	FAT-FREE CONDITION	WEIGHT OF CUTS
				grams
Fat of loin	0.016	0.286	0.073	3188
Fat of round	0.027	0.190	0.068	2278
Fat of rib	0.029	0.266	0.083	761
Composite	0.105	0.664	0.132	
Lean of fat and chuck and neck	0.129	0.762	0.144	24535
Lean of rib	0.139	0.908	0.153	6016
Lean of loin	0.152	0.979	0.169	12917
Lean of round	0.154	0.777	0.157	16946

The above tables referring to steer No. 523, show that the fresh samples contain a little more phosphorus than the corresponding cuts from steer No. 504. The relation does not hold though when the results are compared on a fat free basis; the average results of both only differing in the third place. This is of some interest in view of the fact that No. 504 had been well fed all his life, while No. 523 had had only a medium ration. However the former produced a carcass that graded *Prime* while the latter's carcass graded *No. 3*.

It will be noticed in Tables X and XI referring to No. 525 that the lean of the round and loin cuts contains about the same amounts of soluble phosphorus when compared on either the fresh sample, or the same reduced to a fat free basis. When calculated to a dry and fat free condition the soluble phosphorus in the round is considerably increased over that of the lean loin and other cuts.

Steer No. 525 was a thin animal having been so fed that he gained only one-half pound daily during his life of two years. When slaughtered he was thin, the carcass grading as a *good canner*. Or in other words, the carcass would be used by the packers for dried beef, corned beef, etc., the quality not being good enough for sale in the retail market as fresh beef.

TABLE X.

Partial Composition of Selected Cuts from Steer No. 525.

LAB. NUMBER	DESCRIPTION OF SAMPLE	TOTAL SOLUBLE SOLIDS	WATER	FAT	SOLUBLE PHOSPHORUS Total	SOLUBLE PHOSPHORUS Total Water and Fat-Free	SOLUBLE PHOSPHORUS Total Fat-Free	SOLUBLE PHOSPHORUS Inorganic	SOLUBLE PHOSPHORUS Organic
91168	Lean and fat of chuck and neck	4.77	72.99	8.43	0.133	0.716	0.145	0.040	0.093
91171	Lean of round	5.83	79.85	3.33	0.158	0.933	0.163	0.038	0.120
91172	Fat of round	1.29	40.19	56.25	0.029	0.814	0.066	0.013	0.016
91173	Lean of loin	5.72	76.66	3.35	0.159	0.795	0.164	0.031	0.128
91174	Fat of loin	1.12	25.58	70.75	0.019	0.518	0.065	0.007	0.012
91175	Lean of rib	4.71	70.51	8.68	0.141	0.677	0.154	0.019	0.122
91176	Fat of rib	1.77	30.36	61.43	0.032	0.389	0.083	0.013	0.019
91186	Composite of leans and fats exclusive of above samples*	3.71	65.43	17.29	0.112	0.648	0.135	0.028	0.084

*Obtained in the same way as noted under Table VIII.

TABLE XI.

Cuts of Steer No. 525 Arranged According to Percentage of Total Soluble Phosphorus. Referred to Different Conditions of the Cuts.

DESCRIPTION OF SAMPLE	FRESH CONDITION	WATER AND FAT-FREE CONDITION	FAT-FREE CONDITION	WEIGHT OF CUTS
				grams
Fat of loin	0.019	0.518	0.065	1879
Fat of round	0.029	0.814	0.066	981
Fat of rib	0.032	0.389	0.083	332
Composite	0.112	0.648	0.135	
Lean and fat of chuck and neck	0.133	0.716	0.145	17912
Lean of rib	0.141	0.677	0.154	5833
Lean of round	0.158	0.933	0.163	13762
Lean of loin	0.159	0.795	0.164	9355

Discussion of the Data.

The average per cents, disregarding the weights of the cuts, of total soluble phosphorus reduced to a fat free basis, for the loin rib and round fats of steers 504, 523 and 525 were 0.074, 0.071 and 0.071 respectively; the lean portion of the loin, rib and round contained 0.165, 0.160 and 0.160. The soluble phosphorus in the same lean cuts when reduced to a moisture and fat free condition averages for the three animals as follows, 0.702. 0.888 and 0.802, named in the same order as above.

The average per cents of the soluble phosphorus in the fats calculated according to weight are as follows:

FRESH SUBSTANCE. Round fats, 0.015 per cent; rib fats 0.016 per cent; loin fats, 0.015 per cent.

WATER AND FAT FREE SUBSTANCE. Round fats, 0.298 per cent; rib fats, 0.282 per cent; loin fats 0.411 per cent.

The soluble phosphorus of the lean portions averaged in the same way as the preceding, appears as follows:

FRESH SUBSTANCE. Lean rounds, 0.169 per cent; lean loins, 0.135 per cent; lean ribs, 0.133 per cent.

WATER AND FAT FREE SUBSTANCE. Lean rounds, 0.856 per cent; lean loins, 0.693 per cent; lean ribs, 0.715 per cent.

F. W. Woodman, of this laboratory has shown[1] that the fat ether soluble, extracted from bones contained but 0.003 per cent total phosphorus.

The above demonstrates very clearly that the phosphorus in beef flesh is found chiefly in the muscular or connective tissue.

A comparative study cannot be made of the forms of phosphorus found in the water extracts prepared from the three animals because soluble inorganic phosphorus was determined in samples from steer No. 504 by the Emmett and Grindley method. It has been demonstrated, page 491, that their method gives results which are too high .

It is possible that the condition of the animal may influence the relative amount of organic phosphorus and it is unfortunate that this point cannot be cleared up here. Another investigation conducted in this laboratory, working on the composite sample from a very thin animal, shows that 26 per cent of the soluble

[1] *Journ. of Ind. and Eng. Chem.*, i, p. 725, 1909.

phosphorus is organic. Cuts from a very fat animal show from 52 to 70 per cent of the soluble phosphorus to be in organic forms. Another fat animal gives even higher figures for this component 72 to 87 per cent.

To avoid variables, the weights of the lean and fat of each cut were added and from the percentage composition, a new per cent for soluble phosphorus was computed which then represented the entire cut. The per cent of soluble phosphorus thus obtained was reduced to a fat free, and moisture and fat free condition. The results of this computation are given in Table XII.

TABLE XII

Soluble Phosphorus in the Cuts Calculated to Fat-Free, and Water and Fat-Free Substances.

Basis of comparison	Fresh substance			Water and fat-free			Fat-free		
Number of steer	504	523	525	504	523	525	504	523	525
Chuck and neck	0.100	0.129	0.133	0.567	0.762	0.716	0.131	0.144	0.145
Round	0.145	0.139	0.149	0.807	0.774	0.934	0.189	0.152	0.157
Loin	0.084	0.125	0.135	0.570	0.923	0.782	0.135	0.163	0.159
Rib	0.097	0.126	0.135	0.630	0.850	0.670	0.147	0.149	0.152
Composite	0.076	0.105	0.112	0.543	0.664	0.648	0.127	0.132	0.135

If we study Table XII, which really includes most of the others pertaining to the analytical results of the animals under discussion, several things become apparent.

The fresh substance of 504 contains less soluble phosphorus than the other animals, the round cut excepted; steer 525 contains more than 504 and 523 in every instance. The composite representing the cheapest cuts in the animal, contains the least, while the round, a medium priced cut, contains the most.

When the soluble phosphorus is expressed in terms of water and fat free substance, the round of 504 and 525 are highest, but the loin contains the most in the cuts from 523. The cheap cuts remain uniformly low.

It is difficult to surmise a reason why the round cut is high in phosphorus. A glance at the total solids which are soluble in water, seems to indicate the same tendency. The round cut consists of muscular tissue subjected to considerable work and

but seldom relaxed; it contains more water than the other cuts and is high in total soluble solids. Such conditions would naturally favor the retention of soluble phosphorus compounds. Moreover it would indicate more muscle plasma than the other cuts, the rib or loin for example.

There remains to be discussed the influence of age and condition. Steers 523 and 525 were the same age, but the soluble phosphorus content, fresh substance, of 523 is uniformly lower. Steer 504 was three months younger, and the cuts from it contain the least soluble phosphorus. A glance at the second division of Table XII will suffice to show there is no age relation on the water and fat-free basis. It is impossible therefore, to say that age has any influence in this respect.

As to condition, it will be recalled that 504 was fat, 523 medium, and 525 thin. On the basis of the fresh meat, it appears that the thin animal contains in each cut more soluble phosphorus than the others; No. 523 more than 504 with the exception of the round cut. On the water and fat free basis it will be observed that the cuts of 525 are above those of 504 except the loin and rib; and if the round is not considered, No. 523 contains more soluble phosphorus than 504.

These results appear to show that the flesh of the thin animal contains more soluble phosphorus than the fat one; also that the quantity decreases with increasing fatness even when reduced to a moisture and fat free basis.

CONCLUSIONS.

(1) A method which involves heating of the solution before precipitation of the inorganic phosphorus, does not yield results which represent the true condition of the soluble forms of phosphorus compounds in cold water extracts of beef.

(2) Soluble organic phosphorus compounds existing in beef and in cold water extracts of the same, are converted into inorganic forms by heat.

a. The change is practically complete when the temperature is maintained at 70° for fifteen minutes.

b. From 52 to 65 per cent of the total phosphorus in cold water extracts is in the organic form, but may be reduced to from 9 to

20 per cent if heated to about 70°, accompanied by a corresponding increase of the inorganic phosphorus.

(3) The round cut of beef contains more phosphorus, in forms which are soluble in cold water, than any of the other cuts.

(4) Phosphorus is found chiefly in the muscular or connective tissue, the fats contain but little.

(5) The flesh of a thin animal contains more soluble phosphorus than that of a fat animal.

(*a*) The quantity decreases with increasing fatness even when it is expressed on a moisture and fat free basis.

A NEW FORM OF EXTRACTION APPARATUS.[1]

By CHARLES WILSON GREENE.

(Received for publication March 17, 1910.)

A new form of extraction apparatus involving the principle of the Soxhlet extractor has been in use by me for several years, and I am led to publish this delayed description because of a number of requests for the description by those who wish to apply it in researches on animal tissues.

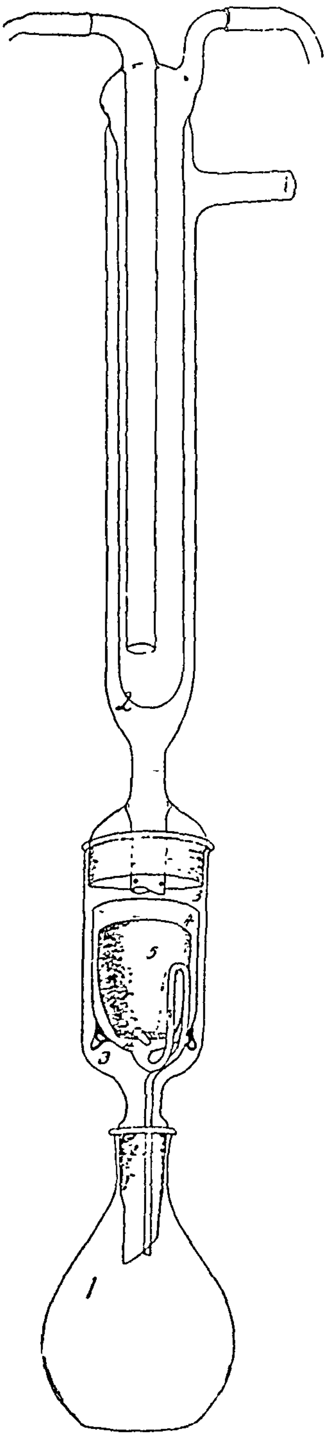
The apparatus was devised for the quantitative analysis of meat samples in order to secure an extraction in hot solvents. In the ordinary Soxhlet extractor the hot vapors pass from the flask through a side tube which reënters above the sample. The extraction is accomplished in a cold, or at best only a lukewarm medium. In the apparatus here described the hot vapors pass up around the cup containing the sample, and the condensate is readily held at or just below the boiling point at all times. The extraction, therefore, proceeds at the boiling temperature. The device has all the advantages of the Soxhlet apparatus in that all parts of the sample are subjected to the actual contact of the hot fluid and this fluid is periodically drained off to be renewed from the condensation of the pure vapors.

Description of Apparatus. The form of the apparatus is illustrated in the accompanying diagram. The apparatus consists of five pieces, which are described as follows:

Number 1 is an ordinary short-necked, narrow-mouthed, flat-bottomed flask which must be pear-shaped for convenience in the transferring of extracting fluids. Its neck is ground to receive the stopper of piece number 3. A most convenient size for this flask is 250 cc. This flask should be made of glass of high resistance as in practice ordinary glass rather has proven to be too breakable. The reflux flask should be made in triplicate.

Number 2 is an ordinary Hopkins condenser, 300 mm. high and 35 mm. in diameter. It has a ground glass stopper at its base. This stopper,

[1] Published by permission of the Commissioner of Fish and Fisheries.



which is ground to fit in piece number 3, is 46 mm. in diameter.[1] The stopper should have a relatively narrow ground surface and be dome shaped as shown in the diagram, as it is found that this form of stopper is more readily manipulated in the transfers that occur in the use of the apparatus. The end of the condenser tube within the stopper is punctured for the reception of a platinum wire where it is desired to suspend a Gooch crucible, paper extraction thimble or other sample container.

Piece number 3, which in practice I have usually called the middle piece, is as its form shows, somewhat like a filtering flask, but with its mouth ground to fit the condenser stopper and its funnel tube ground to fit the reflux flask. This piece must be large enough to receive the siphon cup described below. For the small Gooch a free diameter of 46 mm. is sufficient, and the depth of the bowl should be 100 mm.

Piece number 4 is a shallow cup with siphon tube turned as shown in the figure. This cup should be about 10 to 15 mm. deeper than the height of the Gooch used in extraction—that is about 50 mm. for the 25 cc. Gooch. The siphon should be turned at about the height of 40 mm. from above the bottom of the cup. It is better to have the siphon elbow at the level of the top of the Gooch. In practice one may adjust the height of the Gooch to the different types of material that are to be extracted by inserting a glass rod in the siphon cup on which the Gooch rests. This siphon must be turned snugly against the cup, otherwise it will

[1] The figures are given for a size of extractor adapted to the use of a 25 cc. porcelain Gooch crucible. For the larger Gooch crucible, 40 cc., see the figures given in the table. Special sizes for particular objects are not suggested here.

be broken in placing the cup in and out of the middle piece. The siphon should be constructed of tubing 2 mm. in inside diameter and its end cut so that it extends 1 cm. below the bottom of the stopper of the middle piece when it is set in place. Two small glass rests should be drawn on the floor of the cup, opposite the siphon, to hold the cup off from the wall of the middle piece in order to give free passage to the hot extraction vapors.

Number 5 is an ordinary porcelain Gooch, of the slightly oval form, in which material to be extracted is to be placed. The sizes given above are for a 25 cc. Gooch which conveniently holds a 15 gram sample of meat.

In changing flasks and solvents there is need of some care where, as in alternate alcohol and ether extraction, the boiling points of the solvents vary. Readjustment of the rapidity of heating is called for. There is a certain amount of danger of superheating a solvent of lower boiling point but this can readily be avoided by allowing the apparatus to cool somewhat and the first solvent to be washed out by one or two changes of the second solvent. In alternate alcohol and ether extraction if the change to ether is made while the alcohol in the extraction mass is too hot there is greater danger of an explosion.

This apparatus lends itself to ready adjustment in size and form to meet different purposes for which such apparatus is ordinarily used. The 40 cc. Gooch crucible will hold a meat sample of about 22 grams. For that size of sample, which in certain organs may be preferable to the smaller sample, it is only necessary to vary the size of the middle piece and of the siphon cup to correspond to the increased size of the Gooch crucible. This variation is met by increasing the inside diameter of the middle piece to 50 mm. and the siphon cup to a diameter of 42 mm. The heights in this case remain the same.

For certain types of fractional extraction, one may use a paper extraction thimble instead of the Gooch crucible. A simple variation in sizes to accommodate to the weight of the sample would make the apparatus available for this type of extraction. In practice, the triplicate flasks enable one to shift from one solvent to another by merely exchanging flasks.

I have used this apparatus in sets of five or seven, heating the set over a circular water-bath about 45 cm. in diameter, 12 cm. deep, and supplied with a constant water level 5 cm. from the bottom. The water bath is supplied with sets of porce-

lain rings, also with a wire gauze false bottom located 5 cm. below the top of the bath. When alcohol extraction is taking place, I settle the reflux flask number 1 into the bath some 4 or 5 cm. below the surface of the rings supporting it, letting the flask rest on the wire false bottom. A clean cloth around the set of apparatus tends to hold the steam up around the set. This allows the extraction to proceed at a most effective rate.

In ether extraction, on the other hand, the proper temperature is obtained by raising the apparatus out of the bath and supporting it with one or at most two rings out of the set.

The apparatus has proven very effective in certain extractions for histo-chemical studies. In this case it was desired that the preparation should always be immersed in the solvent and should at no time be allowed to dry. A shallow porcelain shell was set in the siphon cup and the Gooch then set in this shell, The solvent filtered through the Gooch and into the shell overflowing and siphoning off as usual. But the Gooch did not, of course, empty below the level of the top of the shell.

The apparatus has decided advantages by virtue of its dissectible parts. These are more or less interchangeable. When parts are broken they can be renewed with less expense and inconvenience. The cleansing of the apparatus is also facilitated.

Dimensions of the Extractor for 25 cc. and 40 cc. Porcelain Gooch Crucibles.

Piece No. 1. The reflux flask, 250 cc. capacity, pear shape.
Piece No. 2. The Hopkins condenser, 300 mm. long, 35 mm. diameter.
Piece No. 3. The middle piece.

FOR GOOCH NO.	INSIDE DIAMETER AT TOP	LENGTH OF THE CYLINDER	LENGTH OF NECK	LENGTH OF GLASS STOPPERS
	mm.	*mm.*	*mm.*	*mm.*
1.............	46	90	15–20	15–20
2.............	50	90	15–20	15–20

Piece No. 4. Siphon cup:

For Gooch No.	Inside diameter at top	Height clear of turn of siphon	Height of siphon	Length of siphon from top of turn to tip
	mm.	*mm.*	*mm.*	*mm.*
1............	38	50	35–40	100–110
2............	42	55	40–45	110–120

Piece No. 5. The Gooch crucible sizes:

Gooch No.	Capacity	Outside Diameter		Height
		Top	Bottom	
	cc.	*mm.*	*mm.*	*mm.*
1............	25	36	15	40
2............	40	40	25	45

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